

Application Serial No. 09/991,971
Amendment dated 17 September 2003
Reply to Office Action mailed 20 May 2003

AMENDMENTS TO THE SPECIFICATION

Please replace paragraph [0054] with the following amended paragraph:

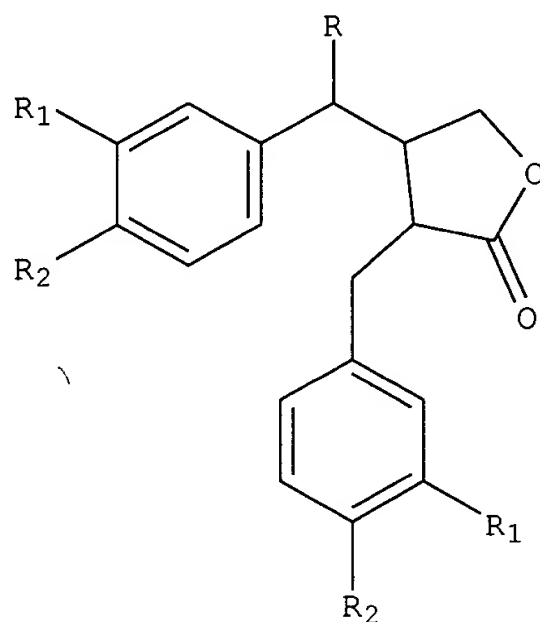
[0054] The results are shown in Figure 3. The solid ~~Solid~~ line represents ~~represent~~ distribution on Fas surface expressing control (untreated) Jurkat cells. The distribution shifted to right (gray area, dotted lines) upon treatment with 50 or 100 μ M hydroxymatairesinol, (histogram I and II), matairesinol ~~enterolactone~~ (III and IV) or enterolactone ~~matairesinol~~ (V and VI). The shift of the graph to the right indicates cells were immunostained for Fas stronger than the control cells thus expressing elevated level of Fas protein on their plasma membrane.

AMENDMENTS TO THE CLAIMS

This Listing of Claims will replace all prior versions, including listings, of claims in the application.

Listing of Claims

Claim 1 (currently amended): ~~A method~~ Method of inhibiting overactivity of phagocytes or lymphocytes in an individual by administering to said individual an effective amount of a lignan, wherein said lignan has the formula



wherein R is H or OH when R₁ is OCH₃ and R₂ is OH or R is H when R₁ is OH and R₂ is H, wherein said lignan is hydroxymatairesinol when R is OH, R₁ is OCH₃ and R₂ is OH, or is matairesinol when R is H, R₁ is OCH₃ and R₂ is OH or is enterolactone when R is H, R₁ is OH and R₂ is H, and

wherein

i) the phagocytes are neutrophils and the lignan is hydroxymatairesinol or matairesinol or a mixture thereof, or

ii) the phagocytes are cells of myeloid origin and the lignan is enterolactone or hydroxymatairesinol or a mixture thereof, or

iii) the lymphocytes are T-lymphocytes and the lignan is hydroxymatairesinol, matairesinol or enterolactone or a mixture thereof.

Claim 2 (original): The method according to claim 1, wherein the phagocytes are neutrophils and the lignan is hydroxymatairesinol or matairesinol or a mixture thereof.

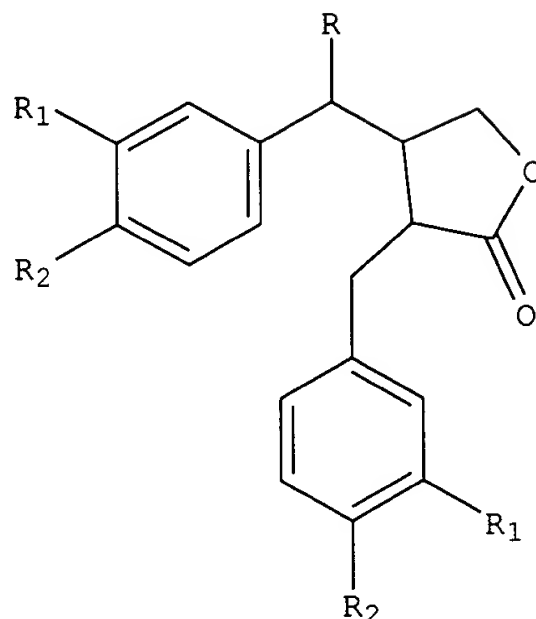
Claim 3 (original): The method according to claim 1, wherein the phagocytes are cells of myeloid origin and the lignan is enterolactone or hydroxymatairesinol or a mixture thereof.

Claim 4 (original): The method according to claim 1, wherein the lymphocytes are T-lymphocytes and the lignan is hydroxymatairesinol, matairesinol or enterolactone or a mixture thereof.

Claim 5 (original): The method according to claim 2, wherein oxidative burst caused by stimulus of the neutrophils is decreased.

Claim 6 (original): The method according to claim 2, wherein the myeloperoxidase activity in converting the reactive oxygen species, released by oxidative burst caused by stimulus of said neutrophils, is decreased.

Claim 7 (withdrawn): Method of treating or preventing an acute ischemia-reperfusion injury or a chronic condition, caused by overactivity of phagocytes or lymphocytes in an individual, said method comprising inhibiting the overactivity of phagocytes or lymphocytes in an individual by administering to said individual an effective amount of a lignan, wherein said lignan has the formula



wherein R is H or OH when R₁ is OCH₃ and R₂ is OH or R is H when R₁ is OH and R₂ is H,
wherein said lignan is hydroxymatairesinol when R is OH, R₁ is OCH₃ and R₂ is OH, or is
matairesinol when R is H, R₁ is OCH₃ and R₂ is OH or is enterolactone when R is H, R₁ is OH and
R₂ is H, and

wherein

- i) the phagocytes are neutrophils and the lignan is hydroxymatairesinol or matairesinol or a mixture thereof, or
- ii) the phagocytes are cells of myeloid origin and the lignan is enterolactone or hydroxymatairesinol or a mixture thereof, or
- iii) the lymphocytes are T-lymphocytes and the lignan is hydroxymatairesinol, matairesinol or enterolactone or a mixture thereof.

Claim 8 (withdrawn): The method according to claim 7, wherein the phagocytes are neutrophils and the lignan is hydroxymatairesinol or matairesinol or a mixture thereof.

Claim 9 (withdrawn): The method according to claim 7, wherein said acute ischemia-reperfusion injury is injury in myocardial infarction, stroke, transplantation, adult respiratory distress syndrome, ischemic heart disease, or endotoxic or hemorrhagic shock.

Claim 10 (withdrawn): The method according to claim 8, wherein said acute ischemia-reperfusion injury is injury in myocardial infarction, stroke, transplantation, adult respiratory distress syndrome, ischemic heart disease, or endotoxic or hemorrhagic shock.

Claim 11 (withdrawn): The method according to claim 7, wherein said chronic condition is rheumatoid arthritis, an allergic condition including also asthma, an inflammatory condition including also inflammatory bowel disease or an inflammatory condition of the skin, HIV, AIDS, psoriasis, Parkinson's disease, Alzheimer's disease, an autoimmune disease, type I or type II diabetes, hypercholesterolemic atherosclerosis, cataract or amyotrophic lateral sclerosis.

Claim 12 (withdrawn): The method according to claim 8, wherein said chronic condition is rheumatoid arthritis, an allergic condition including also asthma, an inflammatory condition including also inflammatory bowel disease or an inflammatory condition of the skin, HIV, AIDS, psoriasis, Parkinson's disease, Alzheimer's disease, an autoimmune disease, type I or type II diabetes, hypercholesterolemic atherosclerosis, cataract or amyotrophic lateral sclerosis.

Claim 13 (withdrawn): The method according to claim 7, wherein the lymphocytes are T-lymphocytes and the lignan is hydroxymatairesinol, matairesinol or enterolactone or a mixture thereof.

Claim 14 (withdrawn): The method according to claim 13, wherein the chronic condition is an allergic or an autoimmune disease, psoriasis, type I ~~and~~ or type II diabetes, rheumatoid arthritis,

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and type I or type II hypersensitivity reactions, asthma, and inflammatory bowel disease, a rejection reaction due to tissue transplantation, atherosclerosis, or multiple sclerosis.

Claim 15 (withdrawn): The method according to claim 7 wherein the phagocytes are cells of myeloid origin, the TNF- α release of which is reduced, and the lignan is enterolactone or hydroxymatairesinol.

Claim 16 (withdrawn): The method according to claim 15, wherein the condition is an inflammatory condition, rheumatoid arthritis, inflammatory bowel disease including also Crohn's disease, Alzheimer's disease, or type I or type II diabetes, atherosclerosis, psoriasis, osteoporosis.

Claim 17 (previously presented): The method according to claim 1, wherein said lignan is hydroxymatairesinol or a mixture of hydroxymatairesinol and matairesinol.

Claim 18 (previously presented): The method according to claim 1, wherein said lignan is hydroxymatairesinol or a mixture of hydroxymatairesinol and enterolactone.

Claim 19 (withdrawn): The method according to claim 7, wherein said lignan is hydroxymatairesinol or a mixture of hydroxymatairesinol and matairesinol.

Claim 20 (withdrawn): The method according to claim 7, wherein said lignan is hydroxymatairesinol or a mixture of hydroxymatairesinol and enterolactone.

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AMENDMENTS TO THE DRAWINGS

The attached sheet of drawings includes changes to Fig. 4A and Fig. 4B. This sheet, which includes Fig. 4A and Fig. 4B, replaces the original sheet including Fig. 4A and Fig. 4B. In Fig. 4A and Fig. 4B, "kontr." has been changed to "control".

Attachment: Replacement Sheet
Annotated Sheet Showing Changes.

REMARKS

The specification has been amended to conform the description at page 10, line 18 with Figure 3.

Fig. 4A and Fig. 4B have been corrected such that "kontr." now reads "control".

Claim 1 has been amended to read "A method" as suggested by the Examiner.

It is submitted that these amendments and corrections do not constitute new matter, and their entry is requested.

The above amendments and correction obviate the objections to the claims and the specification.

The Examiner has rejected claims 1-6 and 17-18 under 35 U.S.C. §112, first paragraph for lack of enablement with respect to the full scope of the claims. The Examiner contends that the specification is enabling only for *in vitro* methods for (1) inhibiting oxidative burst in neutrophils by administering hydroxymatairesinol, (2) inhibiting myeloperoxidase activity in macrophages by administering hydroxymatairesinol and (3) inhibiting Fas induced apoptosis in T-lymphocytes by administering hydroxymatairesinol, matairesinol or enterolactone. Thus, the Examiner contends that the specification is not enabling for any method set forth in the claims for treating any diseases as listed at page 3 of the Office Action. It is submitted that the Examiner is in error in this rejection.

The major emphasis of the Examiner's rejection is the contention that there is no correlation between *in vitro* tests and *in vivo* effects and that the art is unpredictable with respect to such a correlation. It is submitted that there is a good predictability of *in vivo* effects of a drug once corresponding effects have been shown *in vitro*. Copies of the following two articles cited in the specification at page 5, third paragraph, are enclosed for the Examiner's consideration

Dandona et al., *Circulation* **101**:122-124 (2000), demonstrates that the drug carvedilol has antioxidative effects in humans *in vivo*. In the introduction, first paragraph, Dandona states that the same drug (Carvedilol) has been shown to possess antioxidative properties, namely scavenging peroxy and hypochlorous radicals in chemical systems *in vitro* (the *in vivo* study is shown in

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Aruoma, O.I., *Gen Pharmacol.* **28**:269-272 (1997), reference number 3 in the listing at the end of the text).

The second reference, Devaraj et al., *J. Clin. Invest.* **98**:756-763 (1996), demonstrates that alpha tocopherol (Vitamin E) has antioxidative properties in humans *in vivo*. The agent decreases the release of reactive oxygen species, lipid oxidation, interleukin-1-beta secretion and monocyte adhesion to endothelium. This agent had earlier been shown to possess antioxidative properties *in vitro* (see Burton et al. *Ann. NY Acad. Sci.* **570**:7-22 (1989), reference number 55 in the listing at the end of the text).

Both of these two references show, contrary to the Examiner's contention, that there is a clear correlation between test results *in vitro* and effects for the tested drugs *in vivo*. Also enclosed is a copy of the *Merck Manual* index showing the structure of the two compounds. It can be seen that they are, like lignans, rather simple structures. Applicants thus respectively traverse the Examiner's objection as to unpredictability between effects *in vitro* and *in vivo*.

In addition, Applicants offer the following specific remarks with respect to the comments of the Examiner. First, Applicants note that the present claims are directed to a method for inhibiting overactivity of phagocytes and lymphocytes. The claims are not directed to any method for treating any diseases, as asserted by the Examiner in the first paragraph of Point 8 on page 3 of the Office Action. The Examiner has specifically withdrawn claims 7-16 and 19-20 which are directed to methods for treating diseases. These claims were withdrawn by the Examiner as being directed to non-elected inventions. The Examiner has determined that the subject matter of claims 1-6 and 17-18, i.e., a method for inhibiting overactivity of phagocytes or lymphocytes, is patentably distinct from the methods for treating disease. Thus, the subject matter of claims 1-6 and 17-18 is directed to subject matter that is not the treatment of diseases and is patentably distinct from the treatment of diseases. Consequently, the Examiner's contention that the treatment of any disease is not enabled is not appropriate to the claims under consideration. On this basis alone, the rejection must be withdrawn.

With respect to the second paragraph of Point 8 on page 3 of the Office Action, Applicants submit that there is an unpredictability between *in vitro* tests of this kind and *in vivo* results. On the contrary, there is a very good correlation between effects *in vitro* and *in vivo*, as indicated by the above remarks.

With respect to the sixth paragraph of Point 8 on pages 304 of the Office Action, Applicants submit that Table 1 shows both hydroxymatairesinol and matairesinol (i.e., all of the lignans claimed for this aspect) have effect on neutrophils, as shown by oxidative burst and myeloperoxidase activity. Nitecapone and 4-OH-toremifene are both very strong antioxidants. The fact that matairesinol requires a higher dose than the very strong reference compounds for oxidative burst does not mean that matairesinol is ineffective. For myeloperoxidase activity, it should be noted that both hydroxymatairesinol and matairesinol fall between the results of the two strong reference compounds. Therefore, it is shown that both hydroxymatairesinol and matairesinol has effect on neutrophils, measured by oxidative burst and myeloperoxidase activity. Applicants believe it to be unjust if, in order to claim a new use of a compound, they were made to show that the compound shows a stronger property than the strongest known compound for said use.

Example 2 and Figures 2 and 3 show that hydroxymatairesinol, matairesinol and enterolactone all have an effect on T-lymphocytes. Thus, the claimed aspect is fully supported. Example 3 and the Figures 4 show that enterolactone and matairesinol have effects on cells of myeloid origin, in that they decrease TNF-alpha production. It is evident to a skilled artisan that mixtures are also useful once the individual compounds have been shown to work.

The Examiner appears to believe that Table 1 relates to the effect on phagocytes of myeloid origin. This is an erroneous assumption. Table 1 shows the effects on neutrophils. Effects on cells of myeloid origin are shown in Example 3 and in the Figures 4.

With respect to the seventh and eighth paragraphs of page 5 of the Office Action, the tests carried out by Pool-Zobel et al. are not comparable in respect with those of Applicants' invention. Pool-Zobel et al. measures the genetic damage of isolated peripheral lymphocytes. On the contrary,

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Applicants have studied the functional activity of certain cells and the possibility of preventing such activities by use of certain lignans. It is not an aim of the subject invention to destroy cell DNA. Moreover, Pool-Zobel et al. relates to unsorted lymphocytes, not the T-lymphocytes of the present invention.

With respect to the ninth paragraph of Point 8 on page 5 of the Office Action, Applicants submit that the Examiner has formulated a novel test for enablement. The test is to determine whether the Examples would suggest treating a particular disorder, and if not, then looking in the *Merck Manual* to determine if it teaches using the claimed agent for treating the particular disorder. This approach totally ignores the objective enablement of the specification. In addition, Applicants note that if the claimed agent was described in the *Merck Manual* for use in treating a specific disorder, the method would not be new.

In *In re Wright*, 27 USPQ 2d, 1510 (Fed. Cir. 1993), the Federal Circuit made clear that the PTO has the burden of providing a reasonable explanation of why the specification does not enable. There must be some reason to doubt the objective truth of the specification statements. *In re Marzocchi*, 169 USPQ 367 (CCPA 1973). Furthermore, Applicants submit that the Examiner has not provided acceptable evidence inconsistent with the contested statements in the specification.

[I]t is incumbent upon the Patent Office, whenever a rejection on this basis [i.e. doubt of the objective truth of statements in the specification] is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise, there would be no need for the applicant to go through the trouble and expense for supporting his presumptively accurate disclosure.

169 U.S.P.Q. at 370.

Since the Examiner has not presented any scientific evidence or reasons to doubt the objective enablement of the specification for the method for inhibiting the overactivity of phagocytes or lymphocytes by the administration of hydroxymatairesinol, matairesinol and enterolactone, a

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proper case for lack of compliance with the enablement provision of 35 U.S.C. §112, first paragraph has not been established.

In view of the above remarks, it is submitted that claims 1-6 and 17-18 are fully enabled by the specification. Withdrawal of this rejection is requested.


The Examiner has rejected claims 1-6 and 17-18 under 35 U.S.C. §112, second paragraph, for lack of written description. It is submitted that the Examiner is in error in this rejection for the same reasons as detailed above with respect to the enablement rejection. Specifically, the present claims are directed to a method for inhibiting overactivity of phagocytes and lymphocytes. The claims are not directed to any method for treating any diseases, as asserted by the Examiner in the second paragraph of Point 9 on page 6 of the Office Action. The Examiner has specifically withdrawn claims 7-16 and 19-20 which are directed to methods for treating diseases. These claims were withdrawn by the Examiner as being directed to non-elected inventions. The Examiner has determined that the subject matter of claims 1-6 and 17-18, i.e., a method for inhibiting overactivity of phagocytes or lymphocytes, is patentably distinct from the methods for treating disease. Thus, the subject matter of claims 1-6 and 17-18 is directed to subject matter that is not the treatment of diseases and is patentably distinct from the treatment of diseases. Consequently, the Examiner's contention that the treatment of any disease is not adequately described in the specification is not appropriate to the claims under consideration. On this basis alone, the rejection must be withdrawn.

In addition, even if the claims were directed to a method for treating any of the diseases specified by the Examiner, the specification clearly states that the present invention is directed to methods for treating these diseases. There is nothing in the specification which would suggest that Applicants were not in possession of a method for treating these diseases. Applicants also note that the facts of the cited *Lilly* case are inapposite to the presently claimed subject matter. Since the Federal Circuit noted in *Lilly* that the issue of written description is fact specific, *Lilly* is not controlling on the present application. Furthermore, the Examiner is reminded that *Lilly* held that the written description could be satisfied by "words" only.

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In view of the above remarks, it is submitted that claims 1-6 and 17-18 are adequately described by the specification. Withdrawal of this rejection is requested.

In view of the above amendments and remarks, it is submitted that the present claims satisfy the requirements of the patent statutes and are patentable over the prior art. Reconsideration and early notice of allowance are requested. The Examiner is invited to telephone the undersigned in order to expedite prosecution of the present application.

RESPECTFULLY SUBMITTED,					
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<i>Signature</i>				<i>Date</i>	17 September 2003
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Attachments

Dandona et al., *Circulation* **101**:122-124 (2000)

Devaraj et al., *J. Clin. Invest.* **98**:756-763 (1996)

Merck Manual index, pgs. 1879 (Carubicin) and 9932 (Vitamin E)

Carvedilol Inhibits Reactive Oxygen Species Generation by Leukocytes and Oxidative Damage to Amino Acids

Paresh Dandona, MD; Rajaram Karne, MBBS; Husam Ghanim, BS; Wael Hamouda, MS; Ahmad Aljada, PhD; Cesar H. Magsino, Jr, MD

Background—The purpose of this study was to test whether carvedilol has an antioxidant effect in humans *in vivo*.

Methods and Results—We administered 3.125 mg of carvedilol twice daily to normal subjects for 1 week. ROS generation by polymorphonuclear leukocytes and mononuclear cells fell from 314 ± 183.43 and 303 ± 116 mV to 185 ± 157 and 189 ± 63 mV ($P < 0.025$), respectively. *m*-Tyrosine fell from 4.24 ± 0.99 to 4.03 ± 0.97 ng/mL ($P = 0.01$), and *o*-tyrosine fell from 4.59 ± 1.10 to 4.24 ± 0.99 ng/mL ($P = 0.004$) in the absence of a change in phenylalanine concentrations.

Conclusions—We conclude that carvedilol significantly inhibits ROS generation by leukocytes and oxidative conversion of phenylalanine to *m*- and *o*-tyrosine. (*Circulation*. 2000;101:122-124.)

Key Words: carvedilol ■ oxygen ■ leukocytes ■ amino acids

It has been suggested that carvedilol may provide greater benefit than traditional β -blockers in chronic heart failure because of its antioxidant actions that synergize with its nonspecific β - and α -blocking effects.¹ Carvedilol has been shown to inhibit lipid peroxidation of myocardial cell membranes and thus protect endothelial, neuronal, and vascular smooth muscle cells from oxygen radical-mediated injury.² Carvedilol has also been shown to scavenge peroxy and hypochlorous radicals in chemical systems *in vitro*.³ In the only study in humans, carvedilol was shown to have antioxidant actions in patients treated with moderate doses of 25 mg/d as assessed by suppression of ex vivo LDL oxidation and reduction of anti-oxidized LDL antibodies *in vivo*.⁴

Ortho-tyrosine (*o*-tyrosine) and meta-tyrosine (*m*-tyrosine) have recently been shown to be useful markers of oxidative damage to phenylalanine, because they are formed after reactive oxygen species (ROS) attack on phenylalanine. Thus, their concentration is considered to be an index of oxidative damage to amino acids and proteins.

We undertook this study to investigate the effect of carvedilol administration on ROS generation by polymorphonuclear leukocytes (PMNLs) and mononuclear cells (MNCs). We also measured *o*-tyrosine and *m*-tyrosine in plasma as indices of oxidative damage to phenylalanine.

Methods

Eight normal subjects 26 to 33 years old volunteered for the study. The study was approved by the Institutional Review Board of the State University of New York at Buffalo. Written, informed consent was obtained from each subject. None of the subjects were on any medications, including NSAIDs, vitamin E, or other antioxidants.

Fasting blood samples were collected at baseline in tubes with EDTA as an anticoagulant. The subjects were given 3.125 mg of carvedilol PO twice a day for 7 days. On day 8, another fasting blood sample was collected as above. ROS generation by PMNLs and MNCs and levels of *o*-tyrosine and *m*-tyrosine in plasma were measured at baseline and on day 8.

A group of 6 control subjects, not given any drugs, also had 2 fasting blood samples taken 1 week apart without any drug intervention.

Preparation of PMNLs and MNCs

PMNLs and MNCs were prepared, washed, and suspended in HBSS as previously described.⁵

Measurement of ROS Generation

Respiratory burst activity of PMNLs and MNCs was measured by detection of superoxide radical via chemiluminescence.⁶ Five hundred microliters of PMNLs or MNCs (2×10^5 cells) was delivered into a Lumiaggregometer (Chronolog) plastic flat-bottom cuvette to which a spin bar was added. Fifteen microliters of 10 mmol/L luminol was then added, followed by 1 μ L of 10 mmol/L formyl-methionylleucylphenylalanine (FMLP). Chemiluminescence was recorded for 15 minutes (a protracted record after 15 minutes did not alter the relative amounts of chemiluminescence produced by various cell samples). Our method, developed independently, is similar to that published by Tosi and Hamedani.⁷ The interassay coefficient of variation (CV) for this assay is 6%. We have further established that in our assay system, there is a dose-dependent inhibition of chemiluminescence by superoxide dismutase and catalase: superoxide dismutase inhibited chemiluminescence by 82% at 10 μ g/mL, whereas catalase inhibited chemiluminescence by 47% at 40 μ g/mL. Chemiluminescence is also inhibited by diphenyleneiodonium chloride (data not shown), a specific inhibitor of NADPH oxidase, the enzyme responsible for the production of superoxide radicals.⁸ Our assay system is exquisitely sensitive to diphenyleneiodonium chloride at nanomolar concentrations.

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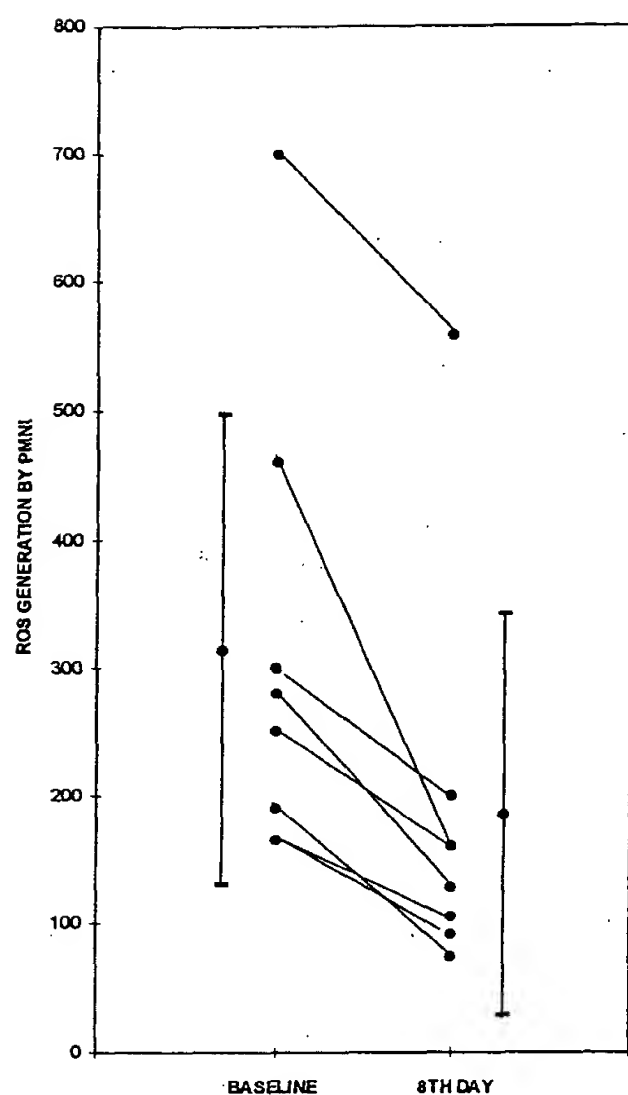


Figure 1. ROS generation by PMNLs before and after 7-day treatment with carvedilol. ROS generation-induced chemiluminescence was measured in millivolts, assayed in 200 000 MNCs in HBSS, and stimulated with FMLP.

Assay of *o*-Tyrosine, *m*-Tyrosine, and Phenylalanine

o-Tyrosine, *m*-tyrosine, and phenylalanine were measured by high performance liquid chromatography using the technique described by Ishimitsu et al.⁹

Statistical Analysis

Comparisons of the ROS generation values at baseline and on day 8 were carried out by Wilcoxon rank sum test, because the distribution of the values was not normally distributed. The values of *o*-tyrosine and *m*-tyrosine before and after carvedilol were compared by paired *t* test.

Results

ROS generation by PMNLs at baseline was 313.75 ± 183.43 mV (mean \pm SD). After carvedilol administration, ROS generation fell to 185.00 ± 156.98 mV. The mean fall was $43.75 \pm 15.31\%$ (range, 20% to 65%; $P=0.025$) (Figure 1). The mean ROS generation by PMNLs in the control group was 544 ± 85 mV at baseline, and it was 515 ± 80 mV 1 week later ($P=NS$).

ROS generation by MNCs at baseline was 302.50 ± 115.70 mV (mean \pm SD). After carvedilol administration, ROS generation fell to 189.25 ± 63.09 mV. The mean fall was $34.77 \pm 14.58\%$ (range, 21% to 57%; $P=0.025$) (Figure 2). Mean ROS generation by MNCs in control subjects was 336 ± 45 mV at baseline and 330 ± 42 mV a week later ($P=NS$).

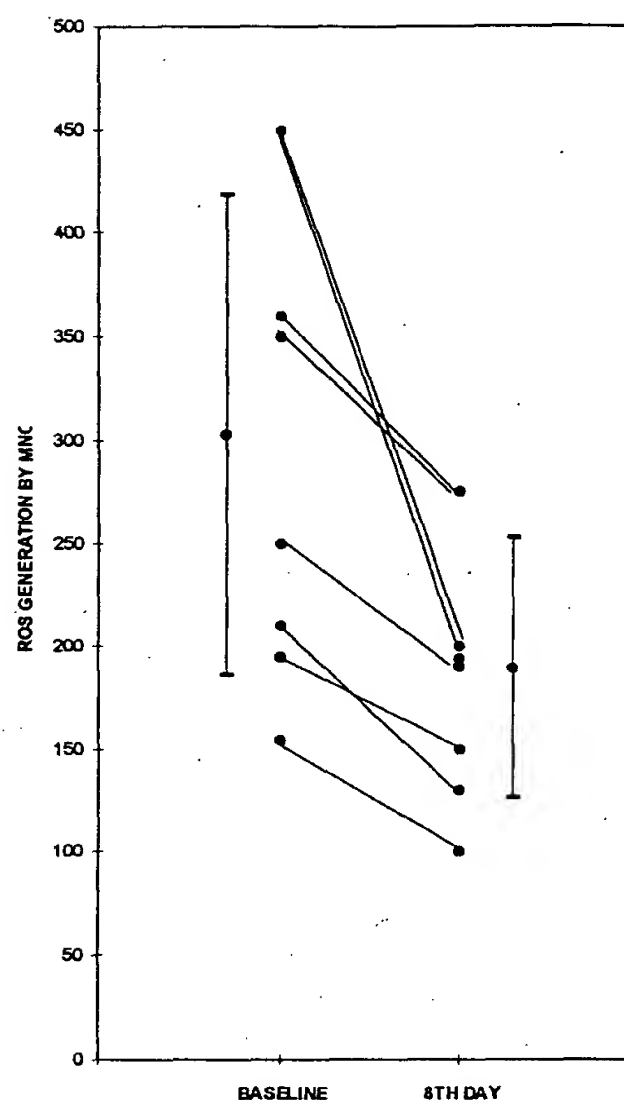


Figure 2. ROS generation by MNC (in millivolts).

Plasma phenylalanine concentration did not change after carvedilol. Plasma *m*-tyrosine concentration fell from 4.24 ± 0.99 to 4.03 ± 0.97 ng/mL ($P=0.01$). The ratio of *m*-tyrosine to phenylalanine changed from 0.35 ± 0.07 to 0.33 ± 0.07 mmol/mol phenylalanine ($P=0.005$).

Plasma *o*-tyrosine concentration fell from 4.59 ± 1.10 to 4.24 ± 0.90 ng/mL ($P=0.004$). The ratio of *o*-tyrosine to

TABLE 1. Plasma *m*-Tyrosine and *o*-Tyrosine Concentrations (ng/mL) and Phenylalanine Concentrations (μ g/mL) Before and After 7 Days of Treatment With 6.25 mg Carvedilol

Subject	<i>m</i> -Tyrosine		<i>o</i> -Tyrosine		Phenylalanine	
	Baseline	Day 8	Baseline	Day 8	Baseline	Day 8
1	4.20	4.02	4.41	4.36	11.68	11.43
2	4.19	3.75	3.93	3.69	10.98	10.93
3	3.90	3.56	5.46	4.69	10.65	10.23
4	6.13	5.77	6.56	5.80	12.56	12.35
5	4.54	4.67	4.83	4.57	10.10	10.27
6	4.65	4.55	4.87	4.59	11.22	11.56
7	3.66	3.33	3.24	2.98	10.65	10.52
8	2.63	2.60	3.40	3.27	11.35	11.56
Mean	4.24	4.03	4.59	4.24	11.15	11.11
SD	0.99	0.97	1.10	0.90	0.75	0.75
	$(P=0.01)$		$(P=0.04)$		$(P=0.329)$	

TABLE 2. Plasma *m*-Tyrosine/Phenylalanine and *o*-Tyrosine/Phenylalanine Ratios Before and After 7 Days of Treatment With 6.25 mg Carvedilol (mmol/mol Phenylalanine)

Subject	<i>m</i> -Tyrosine/ Phenylalanine		<i>o</i> -Tyrosine/ Phenylalanine	
	Baseline	Day 8	Baseline	Day 8
1	0.33	0.32	0.34	0.35
2	0.35	0.31	0.33	0.31
3	0.33	0.32	0.47	0.42
4	0.45	0.43	0.48	0.43
5	0.31	0.29	0.28	0.26
6	0.38	0.36	0.40	0.36
7	0.41	0.42	0.44	0.41
8	0.21	0.21	0.27	0.26
Mean	0.35	0.33	0.37	0.35
SD	0.07	0.07	0.08	0.07
	(P=0.005)		(P=0.002)	

phenylalanine changed from 0.37 ± 0.08 to 0.35 ± 0.07 mmol/mol phenylalanine ($P=0.002$) (Tables 1 and 2).

Discussion

Our data demonstrate clearly that ROS generation by both PMNLs and MNCs is significantly diminished by the administration of carvedilol at a small dose (6.25 mg/d) to normal subjects. One week of carvedilol treatment resulted in a 44% reduction in ROS generation by PMNLs and a 35% reduction in that by MNCs. In contrast, ROS generation by leukocytes in normal subjects not given any drugs did not change significantly over a period of 1 week. Whether other cells in the body also respond to carvedilol by reducing ROS generation is not clear from our study. If this effect of carvedilol is indeed extended to other cells and tissues, the total oxidative load of individuals on carvedilol should diminish markedly after treatment with this drug. This would be reflected in the reduction of oxidative damage to the body as measured by such indices as *o*- and *m*-tyrosines. Oxidative damage may be an important mechanism underlying several pathophysiological states, eg, atherosclerosis due to oxidative modification of LDL¹⁰; diabetic complications due to oxidative damage of lipids, proteins,¹¹ and DNA¹²; aging due to oxidative damage of proteins; and myocardial damage/loss through oxidative injury.

Our data also demonstrate that *o*-tyrosine and *m*-tyrosine concentrations fall without a change in phenylalanine concentrations. Because *o*- and *m*-tyrosine are formed by ROS attack on phenylalanine, our data indicate that ROS-induced oxidative damage to amino acids and proteins falls in association with the decline in ROS generation by PMNLs and MNCs. This has implications for cellular and extracellular proteins, including enzymes, and their physiological functions. It is possible that apoprotein and lipoprotein molecules may also be involved in ROS-induced damage.

The magnitude of ROS inhibition by MNCs and PMNLs (35% and 44%) was comparable to that observed after administration of vitamin E (1200 IU/d) for 8 weeks, a reduction of $\approx 50\%$ in superoxide radical generation and H_2O_2 production by monocytes.¹³

Carvedilol has been shown to improve outcomes in congestive heart failure by reducing morbidity and mortality and the

rate of hospital admissions.¹ Although the reduction in sudden death in such patients may be a function of the antiarrhythmic effects of carvedilol, the reduction of deterioration of congestive heart failure may be due to its antioxidant effects, possibly through the protection of the myocardium from ROS damage.

The mechanism underlying this inhibitory effect of carvedilol on ROS generation is not clear from our data. Carvedilol has been shown to possess antioxidant properties in various animal models.^{9,10} The experimental data have been focused on carvedilol as a chemical antioxidant. There is only 1 report based on a human study, which demonstrates that the ex vivo oxidizability of LDL prepared from sera of patients treated with carvedilol is significantly diminished.¹¹ Our assay system determines actual ROS generation by leukocytes, thus focusing on the biological antioxidant property of carvedilol. It is possible that carvedilol exerts its antioxidant effect by both chemical and biological mechanisms.

In conclusion, we have demonstrated that carvedilol inhibits ROS generation by PMNLs and MNCs significantly, even after a short-term treatment at a relatively small dose. This reduction in ROS generation probably contributes to the diminished conversion of phenylalanine to *o*- and *m*-tyrosine and to the previously described antioxidant effects and related clinical benefits.

Acknowledgments

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The Effects of Alpha Tocopherol Supplementation on Monocyte Function

Decreased Lipid Oxidation, Interleukin 1 β Secretion, and Monocyte Adhesion to Endothelium

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Abstract

Low levels of alpha tocopherol are related to a higher incidence of cardiovascular disease and increased intake appears to afford protection against cardiovascular disease. In addition to decreasing LDL oxidation, alpha tocopherol may exert intracellular effects on cells crucial in atherogenesis, such as monocytes. Hence, the aim of this study was to test the effect of alpha tocopherol supplementation on monocyte function relevant to atherogenesis. Monocyte function was assessed in 21 healthy subjects at baseline, after 8 wk of supplementation with d-alpha tocopherol (1,200 IU/d) and after a 6-wk washout phase. The release of reactive oxygen species (superoxide anion, hydrogen peroxide), lipid oxidation, release of the potentially atherogenic cytokine, interleukin 1 β , and monocyte-endothelial adhesion were studied in the resting state and after activation of the monocytes with lipopolysaccharide at 0, 8, and 14 wk. There was a 2.5-fold increase in plasma lipid-standardized and monocyte alpha tocopherol levels in the supplemented phase. After alpha tocopherol supplementation, there were significant decreases in release of reactive oxygen species, lipid oxidation, IL-1 β secretion, and monocyte-endothelial cell adhesion, both in resting and activated cells compared with baseline and washout phases. Studies with the protein kinase C inhibitor, Calphostin C, suggest that the inhibition of reactive oxygen species release and lipid oxidation is due to an inhibition of protein kinase C activity by alpha tocopherol. Thus, this study provides novel evidence for an intracellular effect of alpha tocopherol in monocytes that is antiatherogenic. (*J. Clin. Invest.* 1996. 98:756-763.) Key words: atherosclerosis • antioxidants • lipid peroxidation • superoxide

Introduction

To date, much data have accrued to support the concept that oxidatively modified LDL can promote atherogenesis (1-3). In

addition, several lines of evidence (1-3) support the in vivo existence of oxidized LDL. Hence, the role of dietary micronutrients such as alpha tocopherol in preventing LDL oxidation and atherosclerosis assumes great importance. In fact, several lines of evidence support a relationship between low levels of alpha tocopherol and increased cardiovascular morbidity and mortality (4-6) and increased intake with decreased cardiovascular morbidity (7-9). Numerous investigators have shown that alpha tocopherol supplementation decreases LDL oxidative susceptibility as evidenced by an increase in the lag phase of oxidation (10-12). In addition, studies have suggested that alpha tocopherol can have other beneficial effects in atherogenesis. Alpha tocopherol supplementation has been shown to decrease platelet adhesion and aggregation (13, 14). In vitro studies have shown that supplementation of endothelial cells with alpha tocopherol (15) decreases monocyte-endothelial cell adhesion and that alpha tocopherol in vitro decreases smooth muscle cell proliferation (16). Also, alpha tocopherol appears to protect endothelium-dependent vasodilation in cholesterol-fed rabbits (17, 18).

However, to date, there appears to be no data on the role of alpha tocopherol supplementation on monocyte function. The monocyte appears to be a crucial cell in early atherogenesis and fatty streak formation and it has been shown previously that monocytes can oxidatively modify LDL (19). Hence, the aim of this study was to test the effect of alpha tocopherol supplementation on the release of reactive oxygen (ROS)¹ species (superoxide anion and hydrogen peroxide), oxidation of an artificial lipoprotein emulsion, and the release of a potentially atherogenic cytokine, IL-1 β . In addition, the effect of alpha tocopherol enrichment of monocytes on monocyte-endothelial cell adhesion was also investigated.

Methods

Subjects

The subjects for this study were 21 normal healthy controls who fulfilled the following inclusion criteria: (a) no recent infection in the last 6 wk; (b) nonsmokers; (c) no gastrointestinal disorders such as malabsorption; (d) not taking antioxidant supplements, oral contraceptives, hypolipidemic drugs, thyroxine, estrogen, or nonsteroidal antiinflammatory drugs for the past 6 mo; (e) alcohol consumption < 1 oz/d; and (f) normal blood count and renal and hepatic function. The mean age of the subjects was 28 \pm 5.5 yr (range 23-44 yr) and the body mass index was 24.8 \pm 5.6 kg/m² (range 23.2-25.7 kg/m²). The study group comprised of 5 females and 16 males. This study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center.

This work was presented in part at Experimental Biology '96 in Washington, D.C. on April 15, 1996 and was published in abstract form (1996. *FASEB [Fed. Am. Soc. Exp. Biol.] J.* 10:1103).

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1. *Abbreviations used in this paper:* HPF, high power field; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; NF κ B, nuclear factor κ B; PKC, protein kinase C; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

Study design

Monocyte function was studied in 21 subjects at baseline (0 wk), after 8 wk of supplementation with alpha tocopherol (1,200 IU/d), and after a 6-wk washout phase when the alpha tocopherol was discontinued (14 wk). The alpha tocopherol was in the form of D- α tocopheryl acetate capsules and was provided by the Henkel Corporation (La Grange, IL). A washout phase was included to compensate for the omission of a placebo group. The placebo group was omitted because of the possibility of wide interindividual variability with monocyte studies (20). Throughout the study, the subjects were requested to adhere to their usual diet and physical activities. Monocyte function was assessed in terms of (a) release of ROS (superoxide anion and hydrogen peroxide); (b) modification of an artificial lipoprotein (fatty acid-BSA emulsion); (c) release of the cytokine, IL-1 β ; and (d) adhesion of monocytes to human umbilical vein endothelial cells (HUVEC).

Isolation of monocytes

Mononuclear cells were isolated from 120 ml of heparinized fasting venous blood by Ficoll-Hypaque centrifugation as described previously (21). 20 ml of blood (anticoagulated with 10 U/ml heparin) was layered carefully on 15 ml of Ficoll-Hypaque gradient (Sigma Immunochemicals, St. Louis, MO) and centrifuged at 500 g, without brakes at room temperature for 30 min. The mixed mononuclear band was aspirated and the cells were washed three times in phenol red RPMI 1640 medium containing 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine and suspended in a known volume. Leukocyte count was performed on a Coulter counter and then cells were plated ($5-7 \times 10^6$ cells) in 6-well Primaria plates in RPMI 1640 medium. Incubation was carried out at 37°C for 2 h in 5% CO₂/95% air, after which nonadherent cells were removed after washing three times with phenol red-free RPMI 1640 medium. Nonspecific esterase staining revealed that 88.6% of the cells were monocytes (20). All the assays of monocyte activity were undertaken on the day of isolation. All reagents used to assay for monocyte function were tested for endotoxin contamination by the Limulus endotoxin assay and were found to have < 0.06 endotoxin units/ml. The viability of the monocytes was found to be 94% by Trypan blue exclusion (20). LPS was used to activate monocytes as reported previously by Cathcart et al. (20).

ROS

Superoxide. Superoxide anion generation in resting and LPS-activated cells was measured by the SOD-inhibitable reduction of ferricytochrome C (22, 23). Monocytes were incubated in Gey's balanced salt solution (GBSS), pH 7.4, for 60 min at 37°C, with and without SOD (100 μ g/ml, final concentration), in the presence and absence of LPS (100 μ g/ml) and 80 μ M ferricytochrome C in a total volume of 1 ml. The reaction was stopped in melting ice and the absorbance of the supernatant was read at 550 nm. An extinction coefficient of 21.1 mM/cm was used for oxidized versus reduced cytochrome C. Results were expressed as nmol superoxide/min/mg cell protein. Cells were harvested using 0.1 N NaOH and the protein content was measured by the method of Lowry et al. (24).

Hydrogen peroxide. Hydrogen peroxide release in resting and LPS-activated cells was measured by the horseradish peroxidase (HRP)-scopoletin fluorescence method as described by Boveris et al. (25). Hydrogen peroxide is detected by its reaction with HRP, which can oxidize scopoletin with concomitant extinction of its fluorescence at 460 nm when activated at 350 nm. Briefly, the assay system consisted of GBSS, resting or activated cells, 0.01 ml scopoletin (4 μ M, final concentration), 0.2 ml of HRP (0.24 μ M, final concentration) in a final volume of 2.5 ml. Incubation was done at 37°C for 60 min after which the supernatant was aspirated and fluorescence was read at 460 nm. Hydrogen peroxide (0.25–5 μ M) was used as standard and hydrogen peroxide release was expressed as μ M H₂O₂/min/mg cell protein.

Lipoprotein oxidation by monocytes

To study lipoprotein modification by monocytes, resting and activated monocytes were incubated overnight in the presence of an arti-

ficial lipoprotein emulsion. An artificial lipoprotein emulsion was used in order to eliminate the variability that would arise when using three individual LDL samples over a 14-wk period. The artificial lipoprotein emulsion was prepared fresh on the day of the experiment according to the method of Ball et al. (26), with minor modification, using a nominal molar ratio of cholesteryl linoleate (CL), cholesteryl arachidonate (CA), cholesteryl oleate (CO)/bovine serum albumin (fatty acid free) of 60:1. The distribution of CL:CA:CO in mol% was 41:5:17, which corresponds to their distribution in human LDL (27). 50 μ l of artificial lipoprotein emulsion was added to each culture dish in a total volume of 1.0 ml in Ham's F-10 medium, pH 7.4, and incubated for 18 h. Cell-free controls were also set up containing the emulsion in Ham's F-10 medium. At the end of the incubation period, in all experiments, the medium was aspirated out of the dish and centrifuged at 2,000 rpm for 10 min at 4°C and the supernatant was collected to assay for lipid peroxidation by the thiobarbituric acid reactive substances assay (TBARS). TBARS was measured by a modified fluorometric assay as described previously (28), using malondialdehyde as standard. Oxidative modification of the artificial lipoprotein by the monocytes was determined as the differences in absorbance between cells and cell-free controls. TBARS activity was expressed as malondialdehyde equivalents.

Release of ROS and lipoprotein oxidation was also tested in the lymphocyte-rich supernatant obtained after the 2-h incubation step during monocyte isolation since there was lymphocyte contamination (see above).

Release of IL-1 β

The release of IL-1 β was measured in resting and LPS-activated monocytes by ELISA using the human immunoassay kit (Biotrak Immunoassay; Amersham Corp., Arlington Heights, IL) (29).

Monocyte-endothelial cell adhesion

During the course of this study, an assay for monocyte-endothelial cell adhesion was validated and set up. Monocyte-endothelial cell adhesion was carried out in the last eight subjects entered in the study by the Rose-Bengal method (30, 31). Primary cultures of HUVEC were obtained from the laboratory of Dr. N. Oppenheimer-Marks at the University of Texas Southwestern Medical Center (32). Confluent monolayers of HUVEC were washed with 4 vol of incubation medium (DME/F-12 media supplemented with 15% FBS, heparin [90 mg/ml], and endothelial cell growth factor [150 μ g/ml]). One set of HUVEC was incubated with LPS (3 ng/ml) for 3 h at 37°C (33). Monocytes were released from Petri dishes with 10 μ M EDTA in PBS, pH 7.4 (21). After washing with RPMI 1640 medium, monocytes (1×10^5 cells/ml) were incubated in a 400 μ l volume at 37°C in 5% CO₂/95% air with the HUVEC monolayers for 30 min in triplicate. Since adherence of monocytes to the Petri dishes results in some activation, to prevent further manipulation of the monocytes before incubation with endothelial cells, we chose to use the vital stain, Rose-Bengal, instead of chromium labeling of the cells. It has been shown previously that these two methods yield similar results (30, 34). After three washes with PBS to remove unbound cells, 200 μ l of 0.02% Rose-Bengal in PBS was added and incubated at room temperature for 5 min. The excess dye was washed off using three washes of PBS with 10% FCS and then 400 μ l of ethanol/PBS (1:1) was added and left at room temperature for 30 min. Monocyte-endothelial cell adhesion was calculated from the difference in absorbencies at 570 nm between wells that contained monocytes and HUVEC and wells that contained only HUVEC. Adhesion was also verified by counting the cells under a phase-contrast microscope. Cells were counted in five high power fields (HPF) by two individuals and the mean value was taken. Both methods revealed similar data.

The alpha tocopherol content of plasma and monocytes was measured after the medium was aspirated and the cells were harvested. For extraction of alpha tocopherol from cells, 1.0 ml of 0.1 M SDS was added to each dish and alpha tocopherol was extracted twice with 4 ml of hexane after ethanol precipitation (35). The hexane phase was

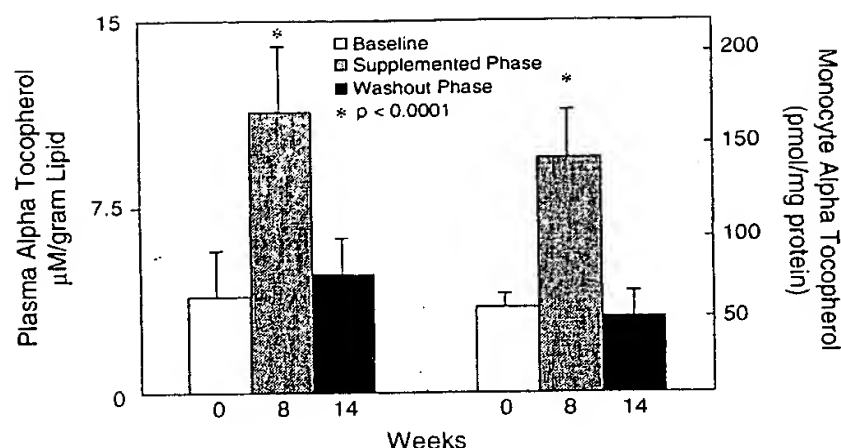


Figure 1. Alpha tocopherol levels in plasma and monocytes. Plasma lipid-standardized and monocyte alpha tocopherol content were measured at 0, 8, and 14 wk after ethanol precipitation and hexane extraction by reversed phase HPLC as described in Methods.

evaporated under nitrogen and reconstituted to 125 μ l with ethanol. Alpha tocopherol was measured after ethanol precipitation and hexane extraction by reversed phase HPLC (36). Plasma alpha tocopherol levels were measured as described previously (36) and the data were lipid standardized as reported previously (12).

Statistical analysis was undertaken to assess the significance of the parameters tested with the help of the biostatistician from General Clinical Research Center. Repeated-measures ANOVA was used to assess differences between baseline, supplemented, and washout phases. Multiple comparisons were performed with (Bonferroni adjusted) paired *t* tests using the 0.01 level of significance to adjust for multiple testing. All data are expressed as mean \pm SD unless stated otherwise.

Results

After alpha tocopherol supplementation, plasma lipid-standardized alpha tocopherol levels were significantly increased when compared with baseline and washout phases (incremental increase, 187.1 ± 14.2 and $136.4 \pm 8.3\%$, respectively, $P < 0.0001$) (Fig. 1). Also, alpha tocopherol supplementation resulted in a significant enrichment of alpha tocopherol within the monocytes when compared with baseline and washout

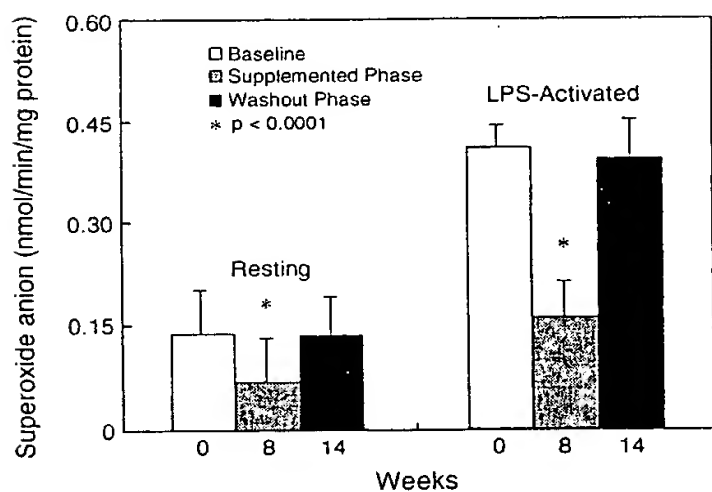


Figure 2. Effect of alpha tocopherol supplementation on superoxide anion release from monocytes. Superoxide anion release was measured in resting and LPS-activated (100 μ g/ml) monocytes at 0, 8, and 14 wk as described in Methods.

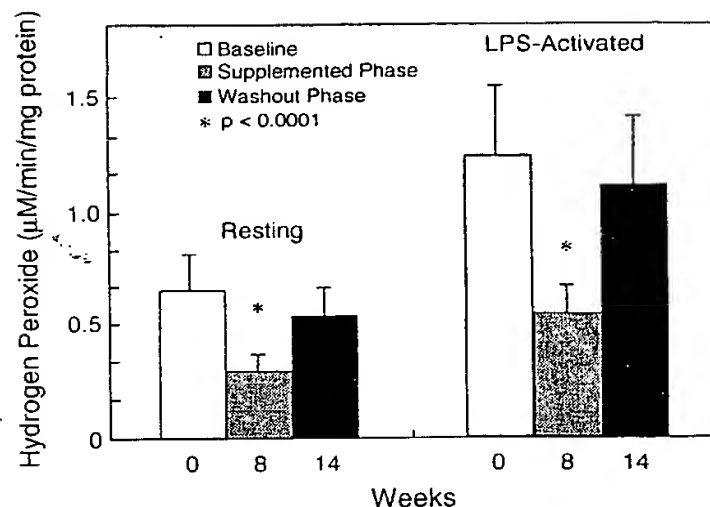


Figure 3. Effect of alpha tocopherol supplementation on hydrogen peroxide release. Hydrogen peroxide from resting and LPS-activated monocytes was measured at 0, 8, and 14 wk as described in Methods.

phases (incremental increase, 169.3 ± 52.3 and $198.9 \pm 51.4\%$, respectively, $P < 0.0001$).

Superoxide anion release from monocytes was assessed at baseline, after supplementation, and at the end of the washout phase. As shown in Fig. 2, there was a significant increase in superoxide anion release from LPS-activated monocytes at 0, 8, and 14 wk when compared with resting cells. Alpha tocopherol supplementation resulted in a significant decrease in superoxide anion release in resting monocytes compared with baseline and washout phases (51.7 ± 3.1 and $50.7 \pm 5.0\%$, respectively, $P < 0.0001$). Also, alpha tocopherol supplementation resulted in a significant decrease in superoxide anion release from LPS-activated monocytes when compared with baseline and washout phases (60.8 ± 6.3 and $59.7 \pm 6.7\%$, $P < 0.0001$). Similar to superoxide anion, there was also a significant increase in hydrogen peroxide release by LPS-activated monocytes ($P < 0.0001$) when compared with resting cells. Alpha tocopherol supplementation resulted in a significant decrease in hydrogen peroxide release from resting cells com-

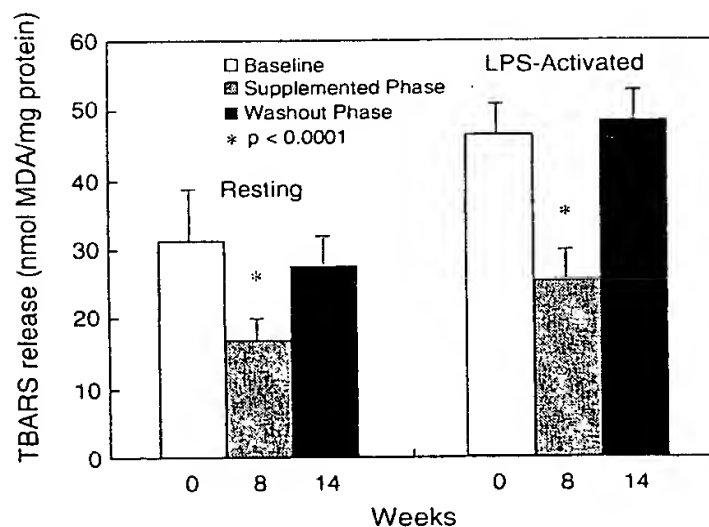


Figure 4. Effect of alpha tocopherol supplementation on lipid oxidation by monocytes. Monocytes, resting and LPS-activated, were incubated overnight with an artificial lipoprotein emulsion containing CL/CA/CO:BSA (60:1). Lipid oxidation by monocytes was measured by assaying for TBARS activity as described in Methods.

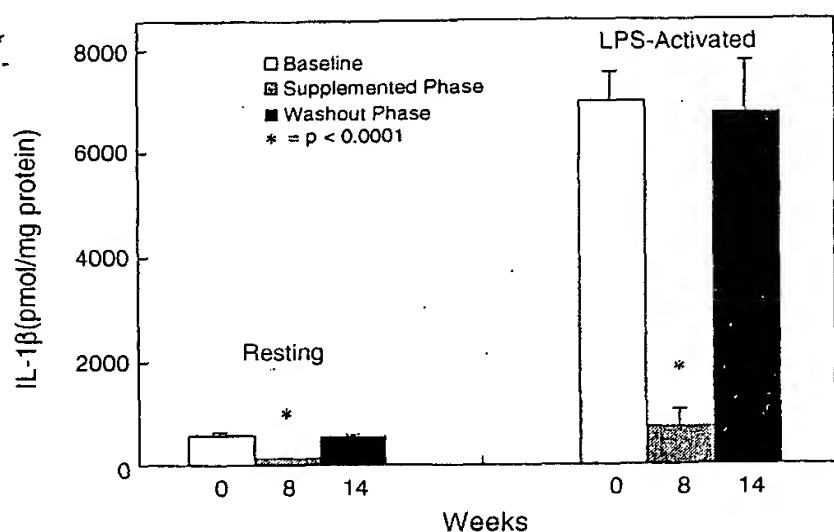


Figure 5. Effect of alpha tocopherol supplementation on IL-1 β release from monocytes. IL-1 β release from resting and LPS-activated monocytes was assayed in the supernatants at 0, 8, and 14 wk by a sandwich ELISA as described in Methods.

pared with baseline and washout phases (46.1 ± 6.7 and $46.5 \pm 8.1\%$, respectively, $P < 0.0001$). As shown in Fig. 3, in LPS-activated monocytes also, alpha tocopherol supplementation produced a significant reduction in hydrogen peroxide release when compared with baseline and washout phases (56.3 ± 12.3 and $51.0 \pm 12.1\%$, respectively, $P < 0.0001$).

Monocyte oxidation of the artificial lipoprotein emulsion was assessed at 0, 8, and 14 wk as depicted in Fig. 4. There was a significant increase in TBARS release after activation with LPS ($60.8 \pm 7.4\%$, $P < 0.0001$). TBARS was significantly re-

duced after alpha tocopherol supplementation in resting monocytes when compared with baseline and washout phases (47.1 ± 12.1 and $39.8 \pm 12.9\%$ reduction, respectively, $P < 0.0001$). In LPS-activated monocytes, alpha tocopherol supplementation also produced a significant decrease in lipid oxidation by the activated monocytes when compared with baseline and washout phases (44.5 ± 14.2 and $46.8 \pm 12.7\%$ reduction, respectively, $P < 0.0001$).

Since the monocyte preparation had some lymphocyte contamination, we determined the contribution of lymphocytes to the indices monitored. Superoxide and hydrogen peroxide release as well as lipid oxidation were tested in the lymphocyte-rich supernatant obtained after the 2-h incubation of the mixed mononuclear cell preparation at 37°C . There was less than a 10% increment in the release of either superoxide or hydrogen peroxide or lipid oxidation in the supernatant.

The effect of alpha tocopherol on IL-1 β release is shown in Fig. 5. Release of IL-1 β was tested in resting and LPS-activated cells. LPS-activated cells showed a 12.1-fold increase in IL-1 β release when compared with resting cells ($P < 0.0001$). The IL-1 β levels after alpha tocopherol supplementation were significantly decreased in resting monocytes when compared with baseline and washout phases (80.2 ± 35.9 and $76.9 \pm 40.1\%$, respectively, $P < 0.0001$). Also, in LPS-activated monocytes, alpha tocopherol supplementation resulted in a significant reduction in IL-1 β levels when compared with baseline and washout phases, respectively (90.3 ± 43.1 and $88.7 \pm 47.5\%$, $P < 0.0001$).

The adhesion of monocytes to confluent HUVEC was also assessed at baseline, after supplementation, and after the washout phases, in the presence and absence of LPS. In spite of the monocytes being activated to some extent by adherence to the Petri dishes, there was a significant increase in adhesion to HUVEC after stimulation with LPS. LPS induced monocyte-endothelial cell adhesion $65.3 \pm 10.5\%$ ($P < 0.0001$) when compared with resting cells. This prior activation of monocytes may also account for the high background adhesion. After alpha tocopherol supplementation, there was a significant decrease in monocyte-endothelial cell adhesion in resting cells (22.5 ± 0.2 and $24.3 \pm 0.2\%$ decrease, respectively, $P < 0.01$) and LPS-activated cells (34.6 ± 0.2 and $36.3 \pm 0.2\%$ decrease, respectively, $P < 0.0003$) when compared with baseline and washout phases (Fig. 6). As shown in the legend to Fig. 6, cell counts per HPF revealed similar findings.

It has been shown previously that alpha tocopherol inhibits protein kinase C (PKC) activity (37,38) and that PKC activity is crucial for superoxide release and LDL oxidation by activated monocytes (39). To gain some mechanistic insights regarding the effect of alpha tocopherol on monocyte function, the effect of the specific PKC inhibitor, Calphostin C, on the parameters of monocyte function was tested. Since Cathcart and Li (39) have shown that 1–10 μM Calphostin C inhibited LDL oxidation by 15–25%, we tested the effect of 0.25 and 0.5 μM Calphostin C on copper-catalyzed LDL oxidation as described previously (12). At both concentrations, Calphostin C had no significant effect on the lag phase of LDL oxidation and maximum amount of oxidation as evidenced by the TBARS and lipid peroxide assay (data not shown). Since Calphostin C was not an antioxidant at 0.25 and 0.5 μM , the effect of these concentrations of Calphostin C on superoxide release, lipid oxidation, IL-1 β release, and monocyte-endothelial adhesion was studied (Table I). Calphostin C (0.5 μM) pro-

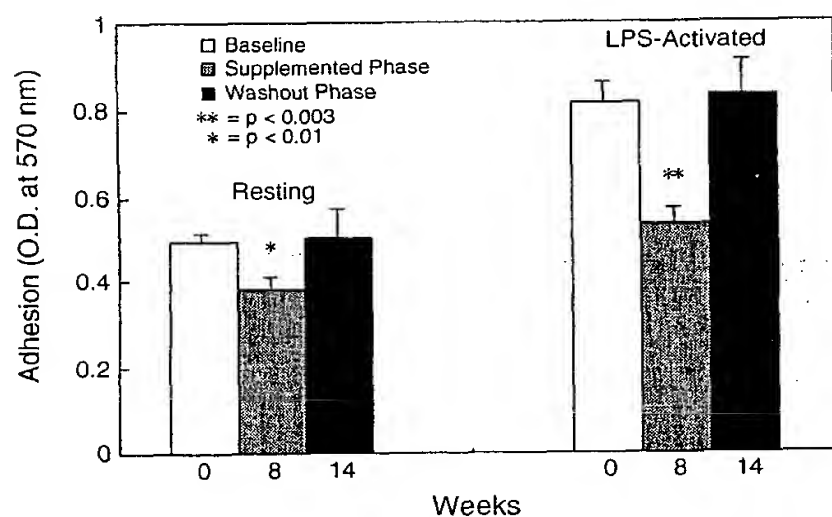


Figure 6. Effect of alpha tocopherol supplementation on monocyte-endothelial cell adhesion. HUVEC were incubated with LPS (3 ng/ml) 3 h before the assay. Monocytes were incubated with control and LPS-activated endothelial cells for 1 h followed by assay of adhesion using the Rose-Bengal method as described in Methods. Control wells consisted of HUVEC only. Cell counts were also done to assess monocyte-endothelial adhesion. For resting cells the number of cells attached per HPF to HUVEC at baseline, after supplementation, and at the end of the washout phase were 49.4 ± 22.9 , 37.1 ± 15.9 , and 52.9 ± 22.7 , respectively. After activation the cells attached per HPF at the three time points were 116.1 ± 52.4 , 76 ± 38.4 , and 120.1 ± 55.6 , respectively. For both resting and activated cells alpha tocopherol supplementation resulted in a significant reduction compared with baseline and washout phases ($P < 0.001$).

Table 1. Effect of Calphostin C on LPS-activated Monocyte Function

	Calphostin C (μ M)		
	0 μ M	0.25 μ M	0.50 μ M
Superoxide anion release (nmol/min/mg protein)	0.44 \pm 0.03	0.41 \pm 0.03	0.19 \pm 0.02*
Lipid oxidation TBARS (nmol/min/mg protein)	31.37 \pm 4.66	23.91 \pm 4.02*	18.27 \pm 2.99*
IL-1 β release (pmol/mg protein)	3075 \pm 378	2762 \pm 178	3177 \pm 301
Monocyte-endothelial cell adhesion Abs 570 nm Cells/HPF	0.44 \pm 0.03 80.3 \pm 17	0.45 \pm 0.01 80.3 \pm 13	0.45 \pm 0.01 78.0 \pm 15

Calphostin C was preincubated with the mixed mononuclear cell preparation for the 2 h and to the monocyte preparation for an additional 30 min. Thereafter, the cells were washed three times in RPMI 1640 before experiments. Values are given as mean \pm SD of three experiments in duplicate. * P < 0.001.

duced a significant decrease in superoxide release from activated monocytes (P < 0.001). Also, both concentrations of Calphostin C produced a significant decrease in lipid oxidation by the monocytes (P < 0.001). However, there was no significant decrease in IL-1 β release or monocyte-endothelial cell adhesion in the presence of Calphostin C.

Discussion

Lower levels of alpha tocopherol have been associated with an increased prevalence of cardiovascular mortality and higher intakes of alpha tocopherol have been shown to be associated with decreased frequency of cardiovascular disease and decreased arterial lesion progression (4–9). Oxidative modification of the intimal lipoproteins is believed to be an essential component of the atherogenic process. In addition to decreasing LDL oxidative susceptibility in vitro, the potential exists that alpha tocopherol could partition in cells such as endothelial cells and monocytes and exert other intracellular effects that could be beneficial. The monocyte-macrophage is one of the crucial cell types in the arterial intima during the genesis of the atherosclerotic lesion and is present during all stages of atherogenesis. There are scanty data on the effect of antioxidant supplementation in vivo on the activity of pivotal cells in atherogenesis, such as endothelial cells and monocytes. The monocyte is the most accessible cell of the artery wall. To this end, we investigated the effect of alpha tocopherol supplementation on release of ROS and lipid oxidation by human monocytes, the release of an atherogenic cytokine, IL-1 β , and monocyte-endothelial cell adhesion.

Monocytes have been shown to induce peroxidation of LDL lipids by generation of ROS such as superoxide and hydrogen peroxide (40). This study has shown that there is a two-fold increase in the release of superoxide anion on activation with LPS and that alpha tocopherol produced a significant reduction in superoxide anion generation from resting and LPS-activated cells. Alpha tocopherol supplementation also significantly decreased hydrogen peroxide release from resting and LPS-activated monocytes. With regards to lipid oxidation, al-

pha tocopherol supplementation also resulted in a significant decrease in oxidation of the artificial lipoprotein emulsion in both resting and LPS-activated monocytes. The monocyte-macrophage is a crucial cell in modifying LDL in lesions. Hence, it is important to characterize LDL modification by monocytes and to see the effect of alpha tocopherol enrichment of monocytes on this process. To minimize assay variability, we chose an artificial lipoprotein which was prepared fresh on the day of the assay instead of LDL which would have to be isolated on three different occasions over 14 wk. We have chosen an artificial lipoprotein emulsion containing cholesteryl linoleate (CL), cholesteryl arachidonate (CA), and cholesteryl oleate (CO)/BSA in the proportion that would normally be present in human LDL (27). The CL/BSA emulsion has been shown to be avidly taken up by macrophages resulting in foam cell formation and ceroid accumulation (26). Mouse peritoneal macrophages and human monocyte-macrophages exposed to CL and CA/BSA rapidly accumulate lipid and oxidize the unsaturated esters (41). Antioxidants such as alpha tocopherol have been found to inhibit this macrophage-mediated lipid oxidation in vitro (42). In the presence of the artificial lipoprotein emulsion, there was a 1.5-fold increase in lipid oxidation by activated monocytes compared with resting cells. Thus, monocyte-mediated lipid oxidation might well contribute to a crucial step in the development of the atherosclerotic plaque. Enrichment of monocytes with alpha tocopherol significantly diminishes the ability of these cells to oxidize lipid and hence could preempt foam cell formation. From this study, it appears that supplementation with alpha tocopherol has dual effects in decreasing LDL oxidation. Numerous groups have shown that alpha tocopherol partitions into the LDL and reduces the oxidative susceptibility of LDL (10–12). The present study indicates that alpha tocopherol supplementation in addition results in enrichment in the monocyte with subsequent decrease in lipid oxidation.

To gain some insights on the effect of alpha tocopherol on monocyte function, we looked at the effect of a specific PKC inhibitor on the parameters of monocyte function studied since Cathcart and Li (39) have shown previously that PKC mediates superoxide release and LDL oxidation by monocytes. The PKC inhibitor, Calphostin C, was chosen since it has been reported to bind efficiently with the regulatory domain rather than the catalytic site and has been shown to be a potent inhibitor of PKC (IC_{50} = 50 nmol) (43). Calphostin C did not show any antioxidant properties or cytotoxicity at 0.25 and 0.5 μ M and therefore these concentrations were used. Calphostin C produced a 51% decrease in superoxide anion release and a 32.3% decrease in lipid oxidation by activated monocytes, which could largely explain the inhibition seen after alpha tocopherol supplementation (59% in superoxide release and 40% decrease in lipid oxidation). Hence, it appears that the inhibition in superoxide anion release and lipid oxidation observed in the subjects after alpha tocopherol supplementation could be attributed to an inhibition of PKC activity rather than a general antioxidant effect.

Increasing evidence suggests that IL-1 β participates either directly or indirectly in growth regulation and formation of atherosclerotic lesions in the arterial wall (44). Also, mRNA encoding for IL-1 β has been found in atherosclerotic lesions (45). Individual cytokines such as IL-1 β have been shown to modulate artery wall cell function, such as the induction of cell adhesion molecule expression (46), such as intercellular adhe-

sion molecule (ICAM) and vascular cell adhesion molecule (VCAM), and stimulation of smooth muscle cell proliferation via PDGF secretion (47). Recently, it has been reported that monokines such as IL-1 and TNF- α can prime PMNs to facilitate ROS production (48, 49). Together, these studies provide support for a potential role for IL-1 β in atherosclerosis and suggest that modulation of cytokine production, e.g., by antioxidant supplementation, would affect the atherosclerotic process. Alpha tocopherol supplementation resulted in a 5- and 10-fold reduction in IL-1 β levels in resting and activated cells. Akesson et al. (50) have shown in vitro that THP-1 cells respond to PMA activation by induction of IL-1 β mRNA and induction of LDL-scavenger receptors. Concomitant with this induction are increased cholesterol esterification and loading by cells. In this in vitro system, probucol and alpha tocopherol were able to inhibit PMA-induced IL-1 β secretion (50). The exact mechanism by which IL-1 release from cells is modulated is not well understood. PMA and other phorbol esters are thought to induce IL-1 activity through activation of cAMP and also via PKC (51, 52). The most potent stimulus for induction and release of IL-1 from monocytes is LPS (53). The lipid-A portion of LPS, which has all the endotoxin activity, is thought to interact with the cell membrane via a putative receptor of target cells and activate immune cell responses, including induction of IL-1 and ROS (54). Since alpha tocopherol is a known chainbreaking antioxidant (55) which provides cell membrane integrity, it could prevent induction of IL-1 release from monocytes by decreasing ROS. Kasama et al. (56) have shown that superoxide stimulates IL-1 release from monocytes and that both SOD and vitamin E inhibit IL-1 release, suggesting that IL-1 activity is enhanced by ROS. Another pathway by which LPS could stimulate IL-1 β release is through the leukotriene pathway. Leukotriene B₄ has been shown to increase IL-1 activity from monocytes (57) and it is possible that alpha tocopherol could reduce the release of IL-1 β from monocytes by decreasing levels of leukotrienes (58). Furthermore, Shapira et al. (59) have shown that LPS-induced IL-1 β production from human monocytes involves both PKC and protein tyrosine kinase. However, no appreciable decrease in IL-1 β release from activated monocytes was observed in our studies using the specific PKC inhibitor, Calphostin C. A plausible mechanism via which ROS stimulate IL-1 release is through activation of transcription factors such as NF κ B (60). Also, components of both lipid and the protein fraction of Ox-LDL have been shown to augment IL-1 release (61, 62). Thus, alpha tocopherol supplementation could conceivably decrease IL-1 β release through an intracellular effect and also by partitioning in LDL and decreasing its oxidative susceptibility. Studies are in progress to elucidate the mechanism(s) by which alpha tocopherol modulates IL-1 β activity from human monocytes.

Studies in nonhuman primates and other animal models have demonstrated that monocyte attachment to endothelial cells, migration, and subendothelial localization are early events in the pathogenesis of atherosclerosis (63). Monocyte enrichment with alpha tocopherol in the supplemented phase resulted in a significant reduction in monocyte-endothelial cell adhesion. Faruqi et al. (15) have observed that when endothelial cells were cultured in media containing alpha tocopherol as a nutritional supplement, there was less agonist-induced monocytic cell adhesion to EC when stimulated with IL-1. The inhibition correlated with a decrease in steady state levels of

E-selectin mRNA and cell surface expression of E-selectin (15). The intracellular signaling events involved in mediating monocyte adhesion to activated EC are not fully defined. PKC activation may be necessary for this process and alpha tocopherol has been shown to inhibit PKC (37, 38). However, in this study, when monocytes were incubated with the PKC inhibitor, Calphostin C, it had no significant effect on monocyte-endothelial cell adhesion. Free radical-mediated injury, either direct or indirect (by generation of oxidized lipoproteins), in the microenvironment of the endothelium is another proposed mechanism by which EC are rendered atherogenic. Biologic response modifiers like IL-1, LPS, and PMA have been shown to induce monocyte-endothelial cell adhesion and these diverse substances may act by intracellular generation of ROS that serve as second messengers in gene activation (64). Immune response has been shown to be associated with expression of endothelial adhesion molecules such as E-selectin, VCAM, and ICAM-1, all of which are activated by the nuclear transcription factor, NF κ B. NF κ B is also activated by oxidative stress, such as hydrogen peroxide and lipid hydroperoxides. Furthermore, antioxidants have been shown to prevent NF κ B activation (64). Suzuki and Packer (65) have shown a concentration-dependent inhibition of NF κ B activation when human Jurkat T cells were incubated with alpha tocopherol acetate or succinate. Thus, alpha tocopherol may exert its attenuating effects at the transcriptional level by inhibiting NF κ B-mediated gene activation in endothelial cells. Since ROS have been shown to increase transcription factors such as NF κ B, it is possible that alpha tocopherol, by inhibiting the release of ROS, results in decreased monocyte-endothelial cell adhesion. However, it should be pointed out that Faruqi et al. (15) failed to demonstrate an effect of alpha tocopherol enrichment of endothelial cells on NF κ B activation in spite of decreasing monocyte adhesion. Adhesion is mediated by integrins that bind to the endothelium, the most important ones being LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and VLA-4 (CD49d/CD29). While VLA-4 binds to VCAM, LFA-1 and Mac-1 bind to ICAM-1 and -2 on the endothelium (66). Future studies will be directed at the effect of alpha tocopherol supplementation on the expression of these counterreceptors on monocytes.

Thus, the novel observations in this study with respect to alpha tocopherol are that in addition to its effects in decreasing LDL oxidation, alpha tocopherol supplementation resulted in an intracellular effect that is antiatherogenic. It decreases the ability of the monocytes to release ROS (hydrogen peroxide and superoxide anion) and significantly reduces lipid oxidation by monocytes. This appears to be mediated by an inhibition of PKC activity. In addition, alpha tocopherol supplementation has other beneficial effects, such as suppression of a potentially atherogenic cytokine, IL-1 β , and inhibition of a crucial event in atherogenesis, monocyte-endothelial cell adhesion. The release of IL-1 β and monocyte-endothelial cell adhesion seem to be regulated via other mechanisms such as activation of transcription factors like NF κ B. The inhibition of IL-1 β release and monocyte-endothelial cell adhesion by alpha tocopherol is possibly due to its antioxidant effect and moderation of the intracellular oxidative stress. Further studies are being carried out to elucidate the mechanism(s) by which alpha tocopherol modulates some of these processes. However, this study provides significant information that strengthens the scientific basis for alpha tocopherol supplementation by clearly demonstrating an intracellular effect in addition to its

protective effect on lipoproteins. Obviously, clinical trials will prove to be the final arbiter in deciding whether alpha tocopherol emerges as an antiatherosclerotic therapeutic modality.

Acknowledgments

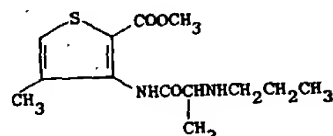
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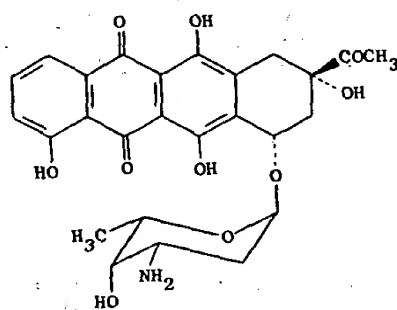
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bp_{0.3} 162-167°.

Hydrochloride, C₁₃H₂₁ClN₂O₃S, Hoe 045, Hoe 40045, Ul-tracain. White, fine crystals, mp 177-178°. LD₅₀ i.v. in mice: 37 mg/kg, R. Muschawek, R. Rippel, loc. cit.

THERAP CAT: Local anesthetic.

1879. Carubicin. 8-Acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-1,6,8,11-tetrahydroxy-5,12-naphthacenedione; (1S,3S)-3-acetyl-1,2,3,4,6,11-hexahydro-3,5,10,12-tetrahydroxy-6,11-dioxo-1-naphthacenyl 3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranoside; 4-O-demethyl-daunorubicin; carminomycin; carminomycin I; karminomycin. C₂₆H₂₇NO₁₀; mol wt 513.52. C 60.81%, H 5.30%, N 2.73%, O 31.16%. Anthracycline antitumor antibiotic, related to daunorubicin and doxorubicin, q.q.v. Isolated from *Actinomyces carminata*: G. F. Gauze et al., *Antibiotiki* 18, 675 (1973); M. G. Brazhnikova et al., *ibid.* 678. Antitumor activity: V. A. Shorin et al., *ibid.* 681. Physico-chemical characteristics, structure: M. G. Brazhnikova et al., *J. Antibiot.* 27, 254 (1974). Pharmacokinetics, pharmacodynamics, toxicity study: L. E. Goldberg et al., *Antibiotiki* 19, 57 (1974). Production: M. G. Brazhnikova et al., U.S.S.R. pat. 508076 (1976 to Inst. Antibiot. Res., USSR), C.A. 86, 15215 (1977). Stereochemistry: *idem*, *J. Antibiot.* 29, 469 (1976). Synthesis from daunomycinone: G. Cassinelli et al., *ibid.* 31, 178 (1978). Molecular pharmacology: V. H. DuVernay et al., *Cancer Res.* 40, 387 (1980). Analysis in human serum: S. E. Fandrich, K. A. Pittman, *J. Chromatog.* 223, 155 (1981). Early clinical studies: L. H. Baker et al., *Cancer Treat. Rep.* 63, 899 (1979). Embryotoxicity and teratogenicity study: I. Damjanov, A. Celluzzi, *Res. Commun. Chem. Pathol. Pharmacol.* 28, 497 (1980).



Hydrochloride, C₂₆H₂₈ClNO₁₀, NSC-180024. Cryst from ethanol/benzene. [α]_D²⁰ +289°. uv max (ethanol): 236, 255, 462, 478, 492 (E_{1%}^{1cm} 300), 510, 525 nm. pK_{a1} 8.00; pK_{a2} 10.16. Sol in water, methanol. Practically insol in other organic solvents. LD₅₀ in mice (mg/kg): 7.3-orally; 1.3 i.v.; 3.7 s.c., L. E. Goldberg et al., loc. cit.

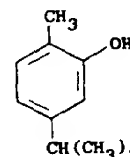
THERAP CAT: Antineoplastic.

1880. Carumonam. [2S-[2α,3α(Z)]]-[[[2-[[2-[(Amino-carbonyl)oxy]methyl]-4-oxo-1-sulfo-3-azetidinyl]amino]-1-(2-amino-4-thiazolyl)-2-oxoethylidene]amino]oxy]acetic acid; (Z)-[[[(2-amino-4-thiazolyl)[[(2S,3S)-2-(hydroxymethyl)-4-oxo-1-sulfo-3-azetidinyl]carbamoyl]methylene]amino]oxy]acetic acid carbamate (ester); (3S,4S)-cis-3-[2-(2-amino-4-thiazolyl)-2-(Z)-carboxymethoxyiminoacetamido]-4-carbamoyloxymethyl-2-azetidinone-1-sulfonic acid. C₁₂H₁₄N₆O₁₀S₂; mol wt 466.40. C 30.90%, H 3.03%, N 18.02%, O 34.30%, S 13.75%. Synthetic monocyclic β-lactam (monobactam) antibiotic. Prepn: S. Kishimoto et al., *Eur. pat. Appl.* 93,376; T. Matsuo et al., U.S. pat. 4,572,801 (1983, 1986 both to Takeda); M. Sendai et al., *J. Antibiot.* 38, 346 (1985). Alternate synthesis: P. S. Manchard et al., *J. Org. Chem.* 53, 5507 (1988). Comparative *in vitro* antimicrobial activity: R. J. Fass, V. L. Helsel, *Antimicrob. Ag. Chemother.* 28, 834 (1985); B. R. Smith et al., *ibid.* 29, 346 (1986); I. M. Hoepelman et al., *Chemotherapy (Basel)* 33, 103 (1987). β-Lactamase stability: R. L. Then, *ibid.* 30, 398 (1984). Pharmacokinetics in humans: E. Weidekamm et al., *Antimicrob. Ag. Chemother.* 26, 898 (1984); C. A. M. McNulty et al., *ibid.* 28, 425 (1985).

Colorless powder. [α]_D²⁵ -45° (c = 1 in DMSO). Disodium salt, C₁₂H₁₂N₆Na₂O₁₀S₂, AMA-1080, Ro 17,2301, Amasulin.

THERAP CAT: Antibacterial.

1881. Carvacrol. 2-Methyl-5-(1-methylethyl)phenol; 2-p-cymenol; 2-hydroxy-p-cymene; isopropyl-o-cresol; isothymol. C₁₀H₁₄O; mol wt 150.21. C 79.95%, H 9.40%, O 10.65%. Found in oil of origanum, thyme, marjoram, summer savory: E. Guenther, *The Essential Oils* vol. 2 (Van Nostrand, New York, 1949) p 503; Carpenter, Easter, *J. Org. Chem.* 20, 401 (1955). Prepn by chlorination of α-pinene with *tert*-butyl hypochlorite: Ritter, Ginsburg, *J. Am. Chem. Soc.* 72, 2381 (1950); from 2-bromo-p-cymol: Strubell, Baumgartel, *Arch. Pharm. Pharmakol.* 291, 66 (1958). Toxicity data: Kochmann, *Arch. Exp. Pathol. Pharmacol.* 161, 196 (1931).

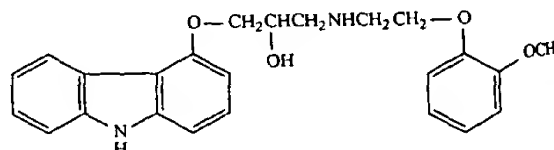


Liquid; thymol odor. d₄²⁰ 0.976; d₂₅²⁵ 0.9751. bp₁₆₀ 237-238°; bp₁₈ 118-122°; bp₃ 93°. mp -0°. n_D²⁰ 1.52295. uv max (95% ethanol): 277.5 nm (log ε 3.262). Volatile with steam. Practically insol in water. Freely soluble in alc or ether. LD orally in rabbits: 100 mg/kg (Kochmann).

USE: As disinfectant; in organic syntheses.

THERAP CAT: Has been used as anti-infective; anthelmintic (Nematodes).

1882. Carvedilol. 1-(9H-Carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)ethyl]amino]-2-propanol; BM-14190; DQ-2466. C₂₄H₂₆N₂O₄; mol wt 406.48. C 70.92%, H 6.45%, N 6.89%, O 15.74%. Nonselective β-adrenergic blocker with vasodilating activity. Prepn: F. Wiedemann et al., *Ger. pat.* 2,815,926; *idem*, U.S. pat. 4,503,067 (1979, 1985 both to Boehringer Mannheim). Pharmacology: G. Sponer et al., *J. Cardiovasc. Pharmacol.* 9, 317 (1987). Clinical pharmacology: E. von Möllendorff et al., *Clin. Pharm. Ther.* 39, 677 (1986); L. X. Cubeddu et al., *ibid.* 41, 31 (1987). HPLC determin in biological fluids: K. Reiff, *J. Chromatog.* 413, 355 (1987). Hemodynamic effects as compared with propranolol, q.v., in hypertension: R. Eggertsen et al., *J. Hypertension* 2, 529 (1984). Clinical trial in hypertension: M. E. Heber et al., *Am. J. Cardiol.* 59, 400 (1987). Clinical efficacy as antianginal: J. C. Kaski et al., *ibid.* 56, 35 (1985); E. A. Rodrigues et al., *ibid.* 58, 916 (1986).



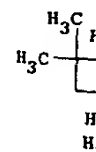
Colorless crystals from ethyl acetate, mp 114-115°. THERAP CAT: Antihypertensive; antianginal.

1883. Carvone. 2-Methyl-5-(1-methylethenyl)-2-cyclohexene-1-one; p-mentha-6,8-dien-2-one; 1-methyl-4-isopropenyl-2-cyclohexen-1-one.

propenyl-150.21. C in caraway Chem. 24, Isolat of d Blanco, R 1480 (196 oils: Kw gingergras (1905). P. 2,796,428 2270 (189-thesis of 1 5856 (195 thesis of a (1958); I. J. L. Simo bridge, 2nd Perfum. Fi

d-Form, [α]_D²⁰ +61.2 l-Form, [α]_D²⁰ -62.4 dl-Form, Practically 1640 mg/kg 327 (1964). USE: As fumery and THERAP C

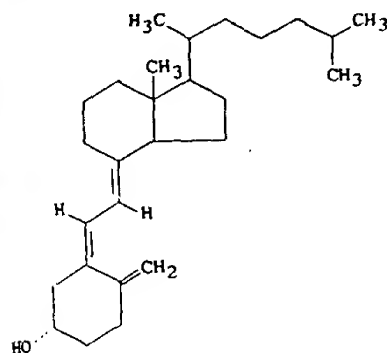
1884. Carvone. 2-Methyl-5-(1-methylethenyl)-2-cyclohexene-1-one; p-mentha-6,8-dien-2-one; 1-methyl-4-isopropenyl-2-cyclohexen-1-one. C₁₀H₁₆O; mol wt 150.21. C 79.95%, H 9.40%, O 10.65%. Found in oil of origanum, thyme, marjoram, summer savory: E. Guenther, *The Essential Oils* vol. 2 (Van Nostrand, New York, 1949) p 503; Carpenter, Easter, *J. Org. Chem.* 20, 401 (1955). Prepn by chlorination of α-pinene with *tert*-butyl hypochlorite: Ritter, Ginsburg, *J. Am. Chem. Soc.* 72, 2381 (1950); from 2-bromo-p-cymol: Strubell, Baumgartel, *Arch. Pharm. Pharmakol.* 291, 66 (1958). Toxicity data: Kochmann, *Arch. Exp. Pathol. Pharmacol.* 161, 196 (1931).



cary

Liquid. H of cloves and [α]_D¹⁵ -5.2°. Dihydrochl Nitrosocylk -98.07°. Isocaryoph:

tadee; Mina D. *Chem. Soc.* 103, 6781 (1981). General review: Inhof-
rol; Dee-Ron-
tol; Hi-Der-
nfron; Rad-
396.63. C 84.35%
n of vitamin D₃
in a suitable
B109, 488 (1960).
of vitamin D₃
Benford, J. Biol.
m. Chem. Soc.
ient of ergosterol
Some U.S. pat.
902,785 (1933);
and 1,871,134 (1934).
al., *Tetrahedron*
l problems: Pre-
in 1930. Stere-
608 (1948).
review: Inhof-
lar, biologically
argocalciferol,
ng rickets in
Suda et al.,
9); *ibid.*, *Biochem.*



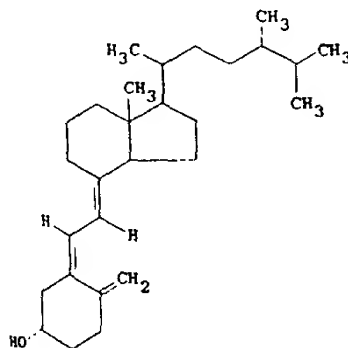
needles from dilute acetone, mp 84-85°. [α]_D²⁰ +84.8° (c = 1.6 in acetone); [α]_D²⁰ +51.9° (c = 1.6 in chloroform).
max (alcohol or hexane): 264.5 nm. (E_{1%}^{1cm} 450-490).
er et al., *J. Am. Chem. Soc.* 67, 609 (1945). Not precipi-
by digitonin (diff from 7-dehydrocholesterol). Practi-
ly insol in water; sol in the usual organic solvents; slightly
in vegetable oils. Oxidized and inactivated by moist air
in a few days. Deterioration of pure crystalline vitamin D₃ is
stable after storage of 1 year in amber evacuated ampuls
refrigerator temps; vitamin D₂ may be kept for 9 months
under the same conditions. Additional stability information:
er, Barlow, *J. Biol. Chem.* 149, 125 (1942). Generally
amin D₃ is considered more stable than vitamin D₂.
Note: Vitamin D₃ is approx as effective as vitamin D₂ in
human and in the rat. It is also fully active in chicks.
amin D₂ is only 1-2 percent as potent for the chick as vi-
amin D₃. Because of this difference it is important that
dry feeds are supplemented with vitamin D₃ rather than
vitamin D₂. One unit (U.S.P. or international) is defined as the ac-
tivity of 0.025 γ of vitamin D₃ contained in the U.S.P. vita-
min D reference standard.

THERAP CAT: Antirachitic vitamin.

THERAP CAT (VET): Nutritional factor (antirachitic).

Sublimes in vac. 9930. Vitamin D₄, 9,10-Secoergosta-5,7,10(19)-trien-3-
ol; 22,23-dihydrovitamin D₃; 22,23-dihydroergocalciferol.
[α]_D²⁵ +82.6° (c = 1.6 in acetone); [α]_D²⁰ +52° (CHCl₃).
bp₁ 200-220°; n_D²⁵ 1.5045. uv max: 294 nm (E_{1%}^{1cm} 71). Prac-
tically insol in water. Freely sol in oils, fats, acetone, alco-
hol, chloroform, ether, other fat solvents. Stable to heat and
alkalies in the absence of oxygen. Not affected by acids up
to 100°. Slowly oxidized by atm oxygen, rapidly by ferric
and silver salts. Gradually darkens on exposure to light.
The I.U. of vitamin E is equal to one mg of standard dl- α -
tocopheryl acetate.

Commercial source: From ergosterol by irradiation with
ultraviolet light in the presence of a trace of magnesium arc.
Chem. Soc., *Perkin Trans. I* 1979, 1290. Its biological
activity seems doubtful.

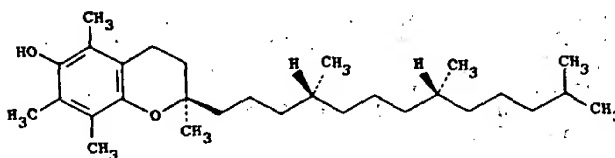


or (antirachitic)
de.

esta-5,7,10(19)-
ovitamin D₃; cho-
l D₃ 1000; Des-
ohyfral D₃; Pro-
De-3-hydroxol;
84.62. C 84.35%
mediates intestinal
bolism and pro-
ormone precursor
st to 25-hydrox-
hydroxycholecal-
108, see Windaus, Guntzel, *Ann.* 538, 122 (1939). [α]_D¹⁸
form. Occurs in
of sepn include
ated by digitonin. Practically insol in water. Sol in the
usual organic solvents except petr ether; slightly sol in vege-
table oils.
Windaus et al., *Z. Phys. Chem.* 149, 125 (1942).
Ann. 533, 118 (1939).
rs 1965, 509.
trahedron Letters
n: V. Malatesta

crystals from dil acetone, mp 96-98°. Originally given as
vitamin D₃, see Windaus, Guntzel, *Ann.* 538, 122 (1939). [α]_D¹⁸
form. Occurs in
of sepn include
ated by digitonin. Practically insol in water. Sol in the
usual organic solvents except petr ether; slightly sol in vege-
table oils.
Windaus et al., *Z. Phys. Chem.* 149, 125 (1942).
Ann. 533, 118 (1939).
rs 1965, 509.
trahedron Letters
n: V. Malatesta

rolin-S; Epsilan; Ephynal; Syntopherol; E-Vimin; Evipherol;
Etavit; Phytogermine; Profecundin; Tokopharm; Viprimol;
Viteolin; Esorb; Vascuals; Covitol; Evion. C₂₉H₅₀O₂; mol wt
430.69. C 80.87%, H 11.70%, O 7.43%. Found largely in
plant materials. Present in highest concns (0.1-0.3%) in
wheat germ, corn, sunflower seed, rapeseed, soybean oils,
alfalfa and lettuce. Natural α -tocopherol is usually found
with β - and γ -tocopherols, q.q.v. Isoln from wheat germ:
Evans et al., *J. Biol. Chem.* 113, 319 (1936). Structure:
Fernholz, *J. Am. Chem. Soc.* 59, 1154 (1937); 60, 700 (1938).
Synthesis: Karrer et al., *Helv. Chim. Acta* 21, 520, 820
(1938); Bergel et al., *J. Chem. Soc.* 1938, 1382; Smith et al.,
Science 88, 37 (1938); Smith, Sprung, *J. Am. Chem. Soc.* 65,
1276 (1943). Recent syntheses: N. Cohen et al., *Helv. Chim.*
Acta 61, 837 (1978); *ibid.*, *J. Am. Chem. Soc.* 101, 6710
(1979); R. Barner, M. Schmid, *Helv. Chim. Acta* 62, 2384
(1979). Abs config of natural α -tocopherol: Mayer et al.,
ibid. 46, 963 (1963). Stereoselective synthesis of the side
chain: C. H. Heathcock, E. T. Jarvi, *Tetrahedron Letters* 23,
2825 (1982). Review of industrial processes: Rubel, *Vita-*
min E Manufacture (Noyes Dev. Corp., Park Ridge, N.J.,
1969). Reviews: *The Vitamins* Vol. 5, W. H. Sebrell, R. S.
Harris, Eds. (Academic Press, New York, 1972) pp 165-317;
J. M. Bieri, P. M. Farrell, *Vitam. Horm. (New York)* 34,
31-75 (1976). Comprehensive description: B. C. Rudy, B.
Z. Senkowski, in *Analytical Profiles of Drug Substances* vol.
4, K. Florey, Ed. (Academic Press, New York, 1975) pp
111-126. Book: *Ann. N.Y. Acad. Sci.* 393, entitled "Vitamin
E: Biochemical, Hematological and Clinical Aspects", B.
Lubin, L. J. Machlin, Eds. (1982) 506 pp. Review of medi-
cal uses: J. G. Bieri et al., *N. Engl. J. Med.* 308, 1063-1071
(1983).



dl-Form, slightly viscous, pale yellow oil. Natural α -
tocopherol has been crystallized, mp 2.5°-3.5°. d₄²⁵ 0.950;
bp₁ 200-220°; n_D²⁵ 1.5045. uv max: 294 nm (E_{1%}^{1cm} 71). Prac-
tically insol in water. Freely sol in oils, fats, acetone, alco-
hol, chloroform, ether, other fat solvents. Stable to heat and
alkalies in the absence of oxygen. Not affected by acids up
to 100°. Slowly oxidized by atm oxygen, rapidly by ferric
and silver salts. Gradually darkens on exposure to light.
The I.U. of vitamin E is equal to one mg of standard dl- α -
tocopheryl acetate.

d-Form, [α]_D²⁵ -3.0° (benzene); [α]_D²⁵ +0.32° (alc).

d- α -Tocopheryl succinate, white powder, mp 76-77°.

α -Tocopheryl nicotinate, C₃₅H₅₃NO₃, Hijuven, Juvela
Nicotinate, Renascin.

USE: As an antioxidant in vegetable oils and shortening.
THERAP CAT: Treatment of vitamin E deficiency.

THERAP CAT (VET): Nutritional factor. Interrelationship
with selenium. (Prevents muscle degeneration, also enceph-
alomalacia and exudative diathesis.) Has been used to pro-
mote fertility.

9932. Vitamin E Acetate. 3,4-Dihydro-2,5,7,8-tetra-
methyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-ol
acetate; 2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-6-
chromanol acetate; α -tocopherol acetate; α -tocopheryl ace-
tate; Alfacol; Contopheron; Detulin; Ecofrol; Econ; E-Ferol;
Endo E Dompé; Ephynal Acetate; Epsilan-M; E-Toplex;
Eusovit; Evipherol; Fertilitvit; Gevex; Juvela; Optovit-E;
Taxofit; Tocopherex; Tocophrin; Tofaxin; Vitagutt. C₃₁H₅₂-
O₃; mol wt 472.73. C 78.76%, H 11.09%, O 10.15%. Prepn
from dl- α -tocopherol and acetic anhydride: Surmatis,
Weber, U.S. pat. 2,723,278 (1955 to Hoffmann-La Roche).
Prepn of d- and l-forms: Robeson, Nelan, *J. Am. Chem.*
Soc. 84, 3196 (1962). Stereoselective synthesis: K.-K. Chan
et al., *J. Org. Chem.* 43, 3435 (1978). Total synthesis of all
eight stereoisomers: N. Cohen et al., *Helv. Chim. Acta* 64,
1158 (1981).

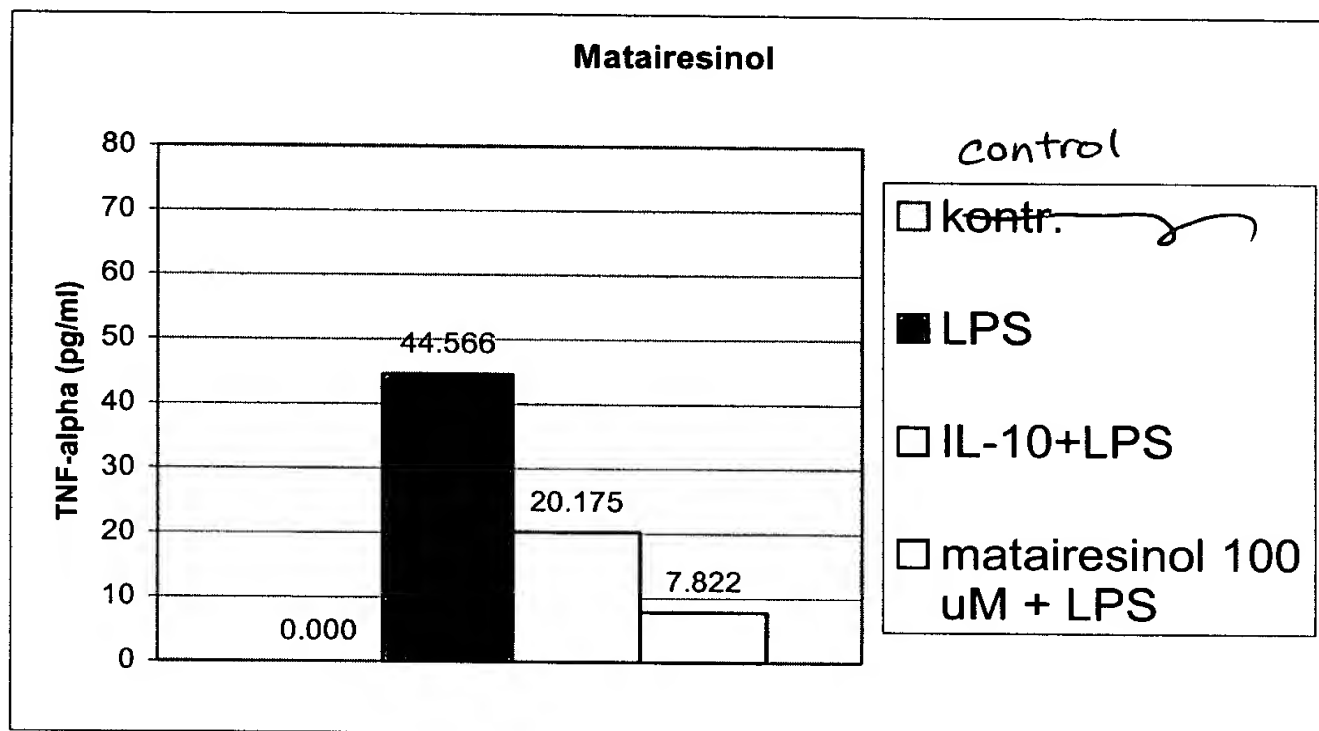


FIG. 4 A

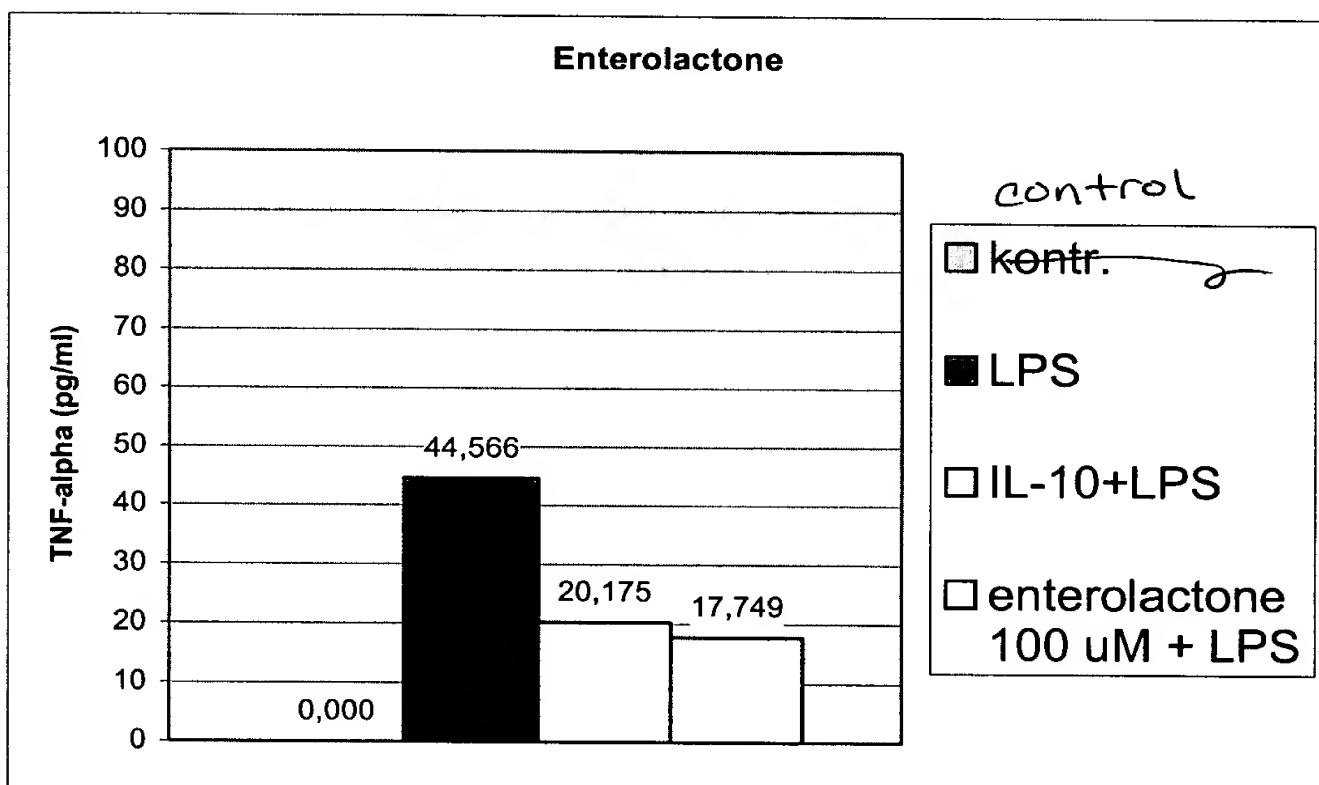


FIG. 4 B